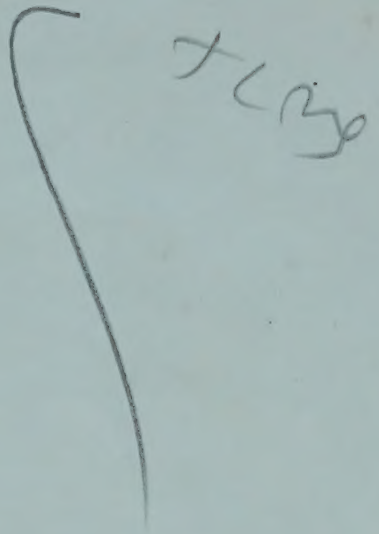
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A TEXTBOOK

OF

BACTERIOLOGY

49

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TO MY MOTHER

PREFACE

Medical Books of any category written in this country are relatively few. The effect of this paucity was strikingly felt during the recent war years. This was one of the impelling reasons behind the present venture which, it is hoped, will in a modest way, help in stimulating the production of Indian publications.

Though there are many admirable textbooks in bacteriology, those treating the subject in all its aspects but confined to the standard befitting the undergraduate student are rare. Exhaustive volumes, like that of Topley and Wilson, are far too elaborate for him. The present volume is also an attempt to remedy this want.

No effort has been spared to treat the subject as clearly as possible consistent with brevity. Repetition is inevitable in a scientific book. Though every attempt was made to minimise it, it has occurred. This is for the most part the result of an endeavour to make each section, as far as possible, self-complete. In this book the application of bacteriology to the clinical and preventive aspects of medicine has been given some prominence, so that the medical student may get a proper perspective of the intimate relationship of the subject to the medical studies in general. It also serves to make the subject more interesting and less academic. A divorce between curative medicine and preventive medicine will spell catastrophe for the general health and well-being of a society. It is hoped that the historical notes added in the beginning of most chapters will make the reading more interesting.

Of the many shortcomings and imperfections that this book may have, I am fully aware of two—the absence of a general historic survey of the subject as a background and an inadequate pictorial representation. The present difficulties in publishing a book are in no small measure responsible for the latter and one hopes that conditions will soon improve. But no explanation nor apology can extenuate a defect.

In the writing of this volume, necessarily many recent works on the subject have been consulted. It is neither possible nor necessary to enumerate them all. But my indebtedness to the following works is too deep to leave them unmentioned: Bergey's "Manual of Determinative Bacteriology", Topley and Wilson's "Principles of Bacteriology and Immunity", the Medical Research Council's "System of Bacteriology", and "Immunity Principles and Application in Medicine and Public Health" by Zinsser Enders and Fothergill.

For the generous help offered in reading the manuscript, I wish to record my deep indebtedness to Dr. V. Govindan Nair, till recently Venereologist and Dermatologist of the K. G. Hospital, Vizagapatam, and to Professor A. Ananthanarayana Iyer of the Madras Medical College. My thanks are specially due to Mr. B. Hanumajee, B.SC., who was for sometime attached to the Bacteriology Department of this College, for the assistance unstintedly rendered in the preparation of this volume, to Dr. K. R. Pandalai for his assistance connected with the proof, and to Mr. Appala Das of this College for the few illustrations and charts. I am also thankful to the members of the staff of the Department of Bacteriology for their ready help and co-operation. I am deeply grateful to the Madras Government and to Col. S. L. Bhatia, Surgeon-General with the Government of Madras, for granting the necessary permission for publishing the book.

VIZAGAPATAM,
1st December 1948.

N. G. PANDALAI.

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A TEXTBOOK OF BACTERIOLOGY

CHAPTER I

GENERAL CHARACTERISTICS OF BACTERIA

Bacteria are regarded as the simplest forms of plant life; but they grow without the aid of chlorophyll. They are extremely minute, unicellular and simple in structure, possessing no definite nucleus. Under suitable conditions, bacteria multiply very rapidly by transverse fission.

Natural Habitat. Bacteria are present everywhere in nature where living conditions exist. They may flourish at any temperature between 0° and 75° C. They are found in large numbers on the body surface of man, animals and plants. Normally they are not found in the living tissues. The very great majority of bacteria live on dead organic matter. Soil, sewage, water and air teem with forms of bacterial life. In most of these situations they are found side by side with other microscopic forms of life like protozoa and fungi. The total microbial population far outnumbers the macroscopic forms of life including plants and animals. Among the myriads of bacteria, only a few are pathogenic; the rest are useful directly or indirectly in the economy of life.

Shape. Bacteria exhibit three fundamental forms: spherical or *coccus*, rod shaped or *bacillus* and spiral. These forms are constant and generally breed true to type, and permanent conversion of one type to another does not occur. Often the generic names of bacteria are explanatory of the shape, for example Staphylococcus in *Staphylococcus aureus* or Bacillus in *Bacillus subtilis*. Within these basic forms variations may occur. Among cocci lanceolate and kidney shaped forms are common. Considerable variations in shape are encountered also among the different species of bacilli; but individuals of the same species exhibit

little tendency to deviate from their basic pattern. The members of some species have parallel sides and rounded ends. Others have parallel sides and square ends. Yet others show bulged out sides, assuming an oval shape; such types, when short, are often referred to as *coccobacilli*. Some bacilli have a fusiform shape with pointed ends. The spiral forms show three subtypes: *vibrio* or curved rod, which, however, represents only a segment of a spiral form, *spirillum* having a rigid spiral body and more than one coil and *spirochæte* with a flexible body consisting of several coils. Among the last named group, there may be even twisting or uneven twisting and straight or curved ends (see Fig. 1).

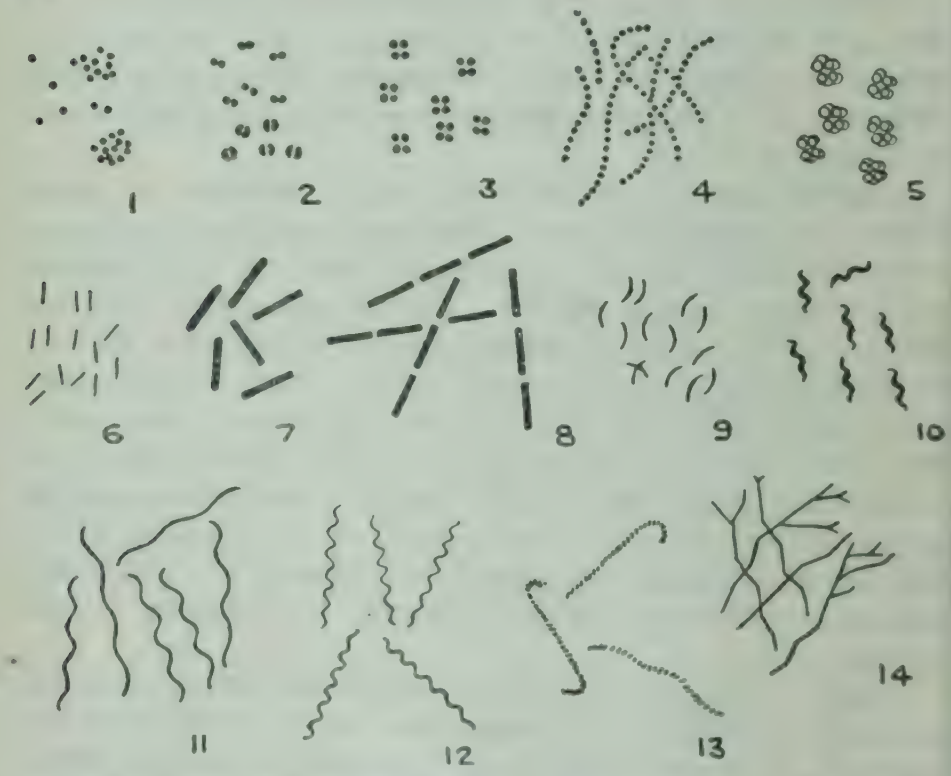


FIG. 1. **Morphology of Bacteria.** 1, staphylococci; 2, diplococci; 3, tetracocci; 4, streptococci; 5, sarcinae; 6-8, different shapes and arrangements of bacilli; 9, vibrio; 10, spirilla; 11-13, spiral organisms; 14, filamentous and branching organisms.

Cell Division and Grouping. During multiplication cells divide. After division the daughter cells may separate completely and appear singly and distributed at random. But a tendency

to remain stuck together after division is a distinctive feature with some species. Among cocci the line of division is in one or more planes. Cocci that divide in one plane and remain apart singly are designated *micrococci*. Those that divide in one plane and break off in pairs are called *diplococci;* organisms that divide in one plane and tend to remain together, forming chains, are called *streptococci*. Those that divide in two planes at right angles to one another and remain in groups of four are called *tetracocci*, and those that divide in three planes and remain grouped together in cubes of eight are called *sarcinæ*. Cocci that divide in all directions and cling together irregularly in the form of grape-like bunches are called *staphylococci* (see Fig. 1). Not all cells in a microscopic field are found to maintain the characteristic grouping. Many individuals appear singly, being broken off from the parent group presumably during manipulation. Bacilli divide only in one plane and this in the transverse direction. Like cocci they may also separate as discrete cells or may remain in pairs, *diplobacilli*, or in chains, *streptobacilli*. Spirochætes also divide only in one plane; no tendency to regular grouping is encountered among them.

Colony Formation. Under suitable conditions of growth, a single bacterial cell planted on a solid medium multiplies rapidly into an aggregate of daughter cells which becomes large enough to be seen by the naked eye. Such masses of bacterial cells are called *colonies*. They exhibit certain morphological characters, particularly when grown on certain culture media, which are distinctive of the species. For example, the colonial characters of the staphylococcus when grown on blood agar are quite different from those of the streptococcus or of the pneumococcus grown on the same medium. The nature of the colony depends largely on the disposition of the daughter cells after division and on the morphology of the individual cells. In some instances, the colony appearances can be brought out more impressively by the incorporation of certain dyes and other substances into the media. This is illustrated by *C. diphtheraie* growing on media containing potassium tellurite. The differentiating peculiarities of bacterial colonies constitute a great aid in the isolation and identification of bacteria. But it should be remembered that such colony characteristics are subject to wide variation, depending upon the conditions of growth. Bacterial variation (*e.g.* S – R

variation), also profoundly affects the morphology of the colonies of the same species.

Size. Different species vary greatly in size; to a less extent it is also true of members of the same species. Variation in shape and size occurring within the same species are sometimes referred to as *pleomorphism*. The dimensions of bacteria are expressed in terms of micromillimetre, abbreviated to micron or μ which is equal to one-millionth of a metre. Bacteria may have any size ranging between $0{\cdot}5\,\mu$ by $0{\cdot}2\,\mu$ and $60\,\mu$ by $5\,\mu$. The pathogenic ones are generally less than $10\,\mu$ in size. The pathogenic spirochætes have much larger measurements. The size of the ordinary pyogenic coccus varies from $0{\cdot}8\,\mu$ to $1{\cdot}2\,\mu$ in diameter. *H. influenzæ*, one of the smallest of the pathogenic bacilli, measures about $0{\cdot}5\,\mu$ by $0{\cdot}2\,\mu$. Some of the pathogens are extremely minute and their size cannot be measured easily. They are called *viruses*.

Structure of the Bacterial Cell. Bacterial cells are highly refractile. Due to the limitations imposed by the extreme smallness of size, no definite structural differentiation can be made out. Certain structures can, however, be readily observed through the optical microscope.

Capsule. Several species of bacteria show a well-defined capsule (see Fig. 2). Some investigators maintain that all bacteria possess capsule and claim to have demonstrated by suitable methods capsules for even those generally classified as non-capsulated. The capsule is supposed to be formed from the superficial layer of the cell. Its presence is not inconsistent with motility. The thickness of the capsule varies in different species and even within the same species. The capsule may be one or two times as thick as the diameter of the cell itself. It is developed best under natural conditions. Under artificial cultivation the capsule does not develop well. The presence of natural proteins, like serum or milk, encourages the development of capsule. Under the microscope the capsule is seen as a white halo round the cell body. It is difficult to stain the capsule unless special methods are employed.

The capsular substance generally consists of carbohydrates; sometimes nitrogen and amino acids may also be present. The capsule is a protective structure for the organism. No definite correlation between virulence and capsule formation has been established. Certain capsulated pathogens, like the pneumococcus

and *B. anthracis*, however, are relatively avirulent in the absence of capsule but highly virulent when they are in the capsulated stage, suggesting a correlation between virulence and the chemical components of the capsule, at least in some cases.

The Cell Membrane. The bacterial protoplasm is contained in a membraneous investment. Many observers look upon this as a definite cell membrane. Others regard it not as a distinct structure but as the thickened and differentiated peripheral layer of the cell substance, similar to the ectoplasm of animal cells, functioning as a cell membrane. In spite of careful investigations, the question has not been finally solved. But recent studies with the electron microscope tend to show that it is a distinct morphological entity. In any case the investment has not the same cellulose structure as in the higher plants. In fact, it has a greater resemblance to the membrane of the animal cell than to that of plants.

The Cell Contents. The cell substance is, of course, protoplasm. It is homogeneous at least in the young growing stage. A definite nucleus is not demonstrable in the bacterial cell. There are several views regarding the nuclear structure. Some of these are: there is a definite nucleus which cannot be demonstrated by the ordinary methods; the cell substance is too elementary and there is no nuclear material at all; the whole cell substance is nuclear matter; bacteria possess both nuclear material and a less differentiated cytoplasm, but both are intimately mixed with each other; the nucleus is in the form of chromatin granules scattered throughout the cytoplasm. Recently, a chromatinic apparatus, which takes the form of one dumb-bell body in each cell unit, has been described by several investigators.

Other Cell Contents. Certain intracellular granules are encountered in many species. They appear as light-refracting in the unstained state. They exhibit a greater affinity for dyes than the rest of the bacterial substance and are, therefore, more intensely stained. There may be one or more of these in the same cell, taking up different positions in the cell contour—polar, bipolar, central or any other. In some species they are well marked in the young cultures and they have some diagnostic value as in the case of the diphtheria bacillus. They are called *metachromatic granules* or *Babes-Ernst bodies* after the names of

their discoverers. Sometimes they are also referred to as *volutin* granules. A high proportion of nucleoprotein is present in their composition. These granules are not degeneration products as they are best seen in young actively growing cultures. They are in some way concerned with cell metabolism and do not take part in cell division or sporulation.

Motility. All cocci are non-motile. Probably the only exception to this is *Rhodococcus agilis*. Many of the bacilli and spirochætes are motile, while the rest are non-motile. The motile ones do not display any antero-posterior polarity; they can move forwards or backwards with equal ease. The motility is of varying character and speed. It may be described as rotatory, wavy, sinuous, darting, etc. The movement may be sluggish, rapid or extremely rapid, but it should be remembered that the speed also is several times magnified. An approximate idea of the rate of movement of bacteria can be gained from the fact that *V. choleræ* has been estimated to attain a maximum speed of 18 centimetres per hour. Again, the quivering molecular movement, exhibited by all very minute organic particles in suspension and known as the *Brownian movement*, should not be confused with motility; the latter is one of movement from place to place executed by

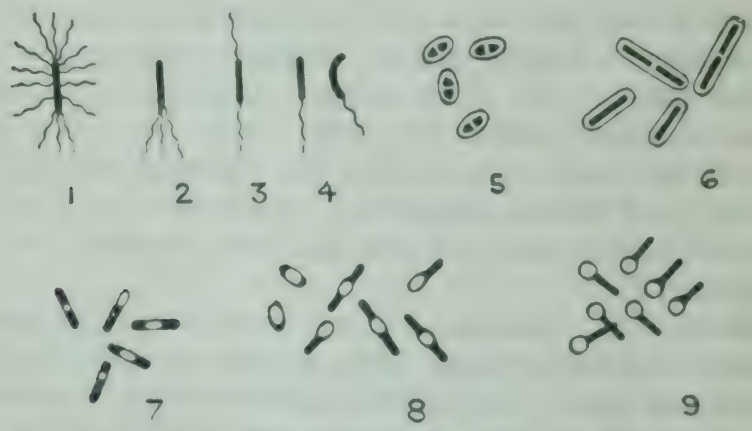


FIG. 2. **Bacterial Flagella, Capsule and Spores.** 1–4, arrangement of flagella – peritrichate, lophotrichate, amphitrichate, monotrichate; 5, capsulated cocci; 6, capsulated bacilli; 7–9, various positions and shapes of bacterial spores.

virtue of life activity. In the former there is no permanent change in position of the moving particle.

Flagella. With the exception of spirochætes, the cells of the motile species are provided with fine hair-like processes projecting out from the body. These appendages, called *flagella*, are the organs of locomotion. Each flagellum may be several times the length of the bacterial body. Their number and position on the cell body vary, but they are constant in the same species. There may be only one or two or several. When there is only one it is placed at one end—*monotrichous* or *monotrichate* (see Fig. 2). There may be one at each end—*amphitrichous*. At one pole, or rarely at both, several may be found in tufts—*lophotrichous*. In some species the flagella are distributed throughout the entire cell surface. This disposition is called *peritrichous*. Opinions differ as to their origin. Some regard them as simple elongations of the cell membrane or the ectoplasm, while others regard them as having their origin in certain endoplasmic granules. The chemical nature of flagella is not definitely known. That there is some difference in this respect between the flagellar substance and the body substance is shown by their antigenic difference. They are easily fragile and difficult to stain. For this reason special methods have to be employed in their staining. It is almost impossible to see them in the unstained state. Flagella are most active in young cultures and are more easily demonstrated in this stage. Older cultures often shed their flagella and lose the power of movement. Under certain conditions, motile bacteria may lose the power of producing flagella and become non-motile for a number of generations. Whether they are able to recover this lost power is not known. Why they behave in this way is also not known. Flagellate ones can be transformed into aflagellate variants by artificial means also. While thus motile bacilli may become non-motile, non-motile ones are never known to acquire the property of producing flagella.

Flagella are not demonstrable in spirochætes. These move by means of an elastic axial filament or a modified fibrillar membrane. They also possess a degree of flexibility never found in the *Eubacteriales*.

Reproduction. Simple binary fission is the usual mode of reproduction among bacteria. This, of course, is asexual in character. Other complex methods of reproduction have been reported as occurring among them. Such should be regarded as not yet established. Division is preceded by an increase in the size of

the cell, as among higher forms of life, the extent of which is constant for each species. Just before division a constriction appears in the middle and ultimately each cell divides into two similar daughter cells. Among the cylindrical and spiral forms, fission occurs at right angles to the long axis, but in coccal forms it occurs in any diameter. The process can be readily observed under the microscope. The daughter cells may separate almost immediately after division or may remain together for varying periods.

The average life of a bacterium is 20–30 minutes. Hence bacteria grow and multiply with enormous rapidity. A single cell may in twenty-four hours give birth to more than fifteen million descendants. The rate of multiplication, however, is profoundly influenced by environmental conditions.

Involution Forms. Constancy of form is usually a marked feature of bacteria during the early stages of growth. But in older cultures aberrant forms soon make their appearance. Such forms are probably developed as the result of unfavourable environmental conditions. They are called *involution* or *degenerative* forms. They may take any shape—thread-like or filamentous, club-shaped and coccoid if young cultures are bacillary and *vice versa*; similarly, elongated forms and giant forms are common in old coccal cultures. Such bizarre forms can be produced at will by growing bacteria under adverse conditions of salt concentration, temperature and others.

Variations During Life Cycle. Rough-smooth variation, formation of filterable forms or of the G. type etc. are encountered during the life cycle of certain bacterial species (Chapter XIV).

Spore Formation. Spore formation is peculiar to certain rod-shaped species. Cocci are not known to sporulate. Most of the anaerobes and some of the aerobes form spores. Only a few of the known pathogens are spore-formers; *Cl. tetani* and *B. anthracis* are examples. As spores are formed within the cells, they are also called *endospores.*

Only one spore develops in one cell. Spores are very refractile, oval or round in shape and bear a definite positional relation with the cell body. If the spore is formed at the end it is called *terminal*, if at the centre of the cell *central* or *equatorial* and when it takes a position midway between the centre and the end of the cell *subterminal* (see Fig. 2). In some species the diameter of the spore does not exceed that of the parent

cell, as in the anthrax bacillus; in others the ripe spore exceeds the diameter of the parent cell, causing a distortion of the morphology of the cell. A vegetative cell with such a large spherical terminal spore will appear like a drum-stick as in *Cl. tetani*. An equatorial large spore bestows a barrel-shaped appearance to the mother cell, and a subterminal spore a racket-shaped appearance.

Spores are formed by the condensation of the protoplasm and the formation of a tough envelope around it. They are very resistant to nocuous agencies, like heat, desiccation or chemical antiseptics. They are difficult to stain and special methods have to be employed for it; but they are easily visible by the indirect staining method. Some bacteria produce their spores early in their life and some late. Some species, for instance the anthrax bacillus, require oxygen for sporulation, while a rigid exclusion of this gas is necessary for others to sporulate. By suitable artificial means spore-producers can be rendered asporogenous, at least for some generations (p. 333). On the spore reaching full development, the parent cell disintegrates and disappears.

Spore formation appears to be a resting stage. It is probably a reaction to adverse conditions, having as its object the preservation of the species. The dormancy enables the spore to remain viable for years or even decades. Spore formation is not a method of reproduction. Each cell forms only a single spore and each spore gives birth to only a single vegetative cell. Thus there is no multiplication.

When favourable conditions are restored, spores germinate into vegetative cells which begin to grow and reproduce in the usual manner. By a simple enlargement a spore may directly assume a bacillary form. The tough spore wall is thinned and stretched but not ruptured or shed. More commonly the new vegetative cell breaks out of the cell wall, discarding the latter. The spore wall may be broken either at one pole or equatorially according to the species.

Staining Reactions. Most bacteria are readily stained by solutions of the aniline dyes. Some of them show a capacity for differential staining and this factor is taken advantage of in diagnosis (p. 66). See Chapter IV.

Chemical Composition of Bacteria. The general chemical composition of bacteria is very much the same as that of other

forms of life. Minor differences are encountered in the chemical structure of different species, but it should be noted that even in the same species the environmental factors, chiefly the composition of the substrate on which the organism grows, appreciably alter the chemical composition quantitatively as well as qualitatively.

As in all forms of life, water is the main ingredient, forming about 70–80 per cent. of the moist cell. The proportion of water in bacteria, however, is slightly less than that in higher living things. Proteins form the major portion of the solid matter. The rest is made up of carbohydrates and small quantities of fats and sometimes inorganic matter.

The ash, or the inorganic residue left after incineration of the cell, constitutes about 2–14 per cent. of the dry weight. But this again is subject to variation even in the same species, depending upon the inorganic content of the surrounding substrate. Phosphoric acid, estimated as P_2O_5, forms 10–45 per cent. of the ash. The phosphorus content of the bacterial cell is very high; most of it is present in the cell in the form of nucleic acid. Sulphur, potassium, calcium, chlorine, and to a less extent, magnesium, iron, silicon and others constitute the rest.

About 50 per cent. of dry weight is carbon. A part of it is present in the cell proteins. The complex polysaccharides, mostly residing in the surface layers of bacteria, are linked with proteins and are in this state responsible for specificity. Capsule, when present, is mostly carbohydrate. Cellulose is not a common constituent of the bacterial cell. Hemicellulose and starch-like materials are sometimes seen, the latter as intracellular granules.

The total nitrogen content of bacteria varies widely. There is not much accord among analysts about the percentage; it may be 8–15 per cent. of the dry weight. Part of the nitrogen exists in the protein of the cell. We know very little about the nature of bacterial proteins. They occur as simple proteins and as complex proteins in union with nucleic acid, with carbohydrates and with lipoids. The importance of this linkage in specificity has been mentioned above. The presence of albumen and globulin has been demonstrated. There is, however, general agreement that nucleoprotein forms a high proportion of the protein matter. In fact, of all the living tissues, except thymus,

bacteria contain the highest proportion of nucleoprotein. Yeast and moulds are also equally rich in this compound.

Fats, lipoids and waxes may be present in bacteria in varying but relatively small quantities. The proportion may sometimes be high, as in the acid-fast bacilli. Estimated as ether-extractible substances, it may vary from 2 to 40 per cent. We know very little about their function in the economy of the bacterial cell.

Growth Curve. The optimum conditions for bacterial growth are present for anything but a short period in a culture medium. Bacterial growth itself very soon brings about significant changes in the environment. The depletion of food and the formation of injurious metabolites, like acids, lytic substances and others, are the main changes. Such changes profoundly influence the rate of multiplication which even under the best of conditions is never uniform except for brief intervals. When expressed graphically—the logarithms of the numbers of bacteria per cubic centimetre plotted against time—the growth curve reveals the presence of several phases of differing activity (see Fig. 3). The period following the transfer of the inoculum to a fresh culture medium is called the *lag phase*. During this period multiplication does not occur, or is at a minimum. There may even be an initial fall in the bacterial count, owing to the death of some of the planted cells. This is probably a period of acclimatisation and preparation; towards the end of this period active multiplication starts and in a very short period the rate of generation reaches its maximum and continues so for a variable time, depending upon the type of the organism and the quality of the culture medium. This period is called the *logarithmic phase*; the rate of increase remains constant and, as cell division occurs in geometric progression, the number of bacteria increases logarithmically with the time. At the end of this period the rate of multiplication begins to slow down, soon reaching a point at which the number of newborn cells equals the number of cells dying. The number of viable count, therefore, remains steady. Hence this period is known as the *stationary phase*. It begins about six to ten hours after inoculation and continues for another five to six hours. It is succeeded by the *phase of decline*, during which the rate of death far outstrips the rate of production. There is, therefore, a progressive fall in the number of viable bacteria until eventually very few or no viable cells are left in the

culture. The increasing death-rate during this phase may be accounted for by the accumulation of injurious growth products and by the fall of oxygen tension in the medium in the case of aerobes.

The duration of these different phases is determined by numerous factors, such as the type of the organism, age and the number of cells in the inoculum, the nature of the culture medium and the temperature and other conditions of experiment. Obviously, therefore, every organism has its own growth curve. Favourable conditions considerably shorten the lag period and the generation time. Certain changes have been observed in the organism during these different phases. Notably, organisms in the logarithmic phase exhibit greater susceptibility to injurious agents, like heat, salt solution and disinfectants, than in the later satges. Organisms in this phase also exhibit greater pathogenicity and virulence; metabolic activity is likewise highest at this period.

Dormancy of Bacterial Cells. Under certain conditions even vegetative bacteria are able to remain dormant for long periods. They are then in a state of suspended animation. Cultures or tissues containing bacteria, when placed in hermetically sealed tubes and preserved in a cool dark place, may remain alive for several years. Advantage is taken of this observation in the preservation of stock cultures.

CHAPTER II

PHYSIOLOGY OF BACTERIA

Simple in structure and often regarded as primitive forms of life, bacteria, nevertheless, have a more complex physiology than what one would expect. The fundamental features of bacterial physiology are, in many respects, similar to those of the higher forms of life. In common with all other forms of life, bacterial life also manifests itself through protoplasm. By exothermic oxidative processes, both cellular constituents and materials of the environment are continuously broken down (catabolism), yielding a constant supply of energy vital for the maintenance of life. Simultaneously with it occurs the complementary phase of assimilation or building up of protoplasm and synthesis of compounds (anabolism) from the available substrate; activities comprising anabolism are energy-consuming or endothermic. These twin components of the chemical aspect of metabolism embrace the entire range of chemical events occurring in the living cell. It should be clearly understood that these two phases are interdependent and concurrently occurring in nature and it is impossible to separate one from the other. As the metabolic process of the structurally simple bacterial cell constitutes most of its activities, the study of bacterial physiology is, for the most part, a study of the biochemistry of bacteria.

In keeping with the primitive structure of the bacterial cell, its physiology exhibits a much lesser degree of specialisation and a greater capacity for adaptation to surroundings than that of the higher forms of life. It is this high degree of physiological flexibility that enables micro-organisms to thrive in such widely diverse situations as soil, water, air and animal and plant surfaces and even bodies, leading to no less diverse phenomena like fermentation, putrefaction, soil fertility and infectious diseases of man and animals.

Nutritional Requirements of Bacteria. Among the many growth requirements of bacteria, a suitable food supply is of

paramount importance. The food requirements of bacteria are of diverse nature and are studied by growing them in artificially prepared or naturally occurring food media called *culture media*. The chemical components that are indispensable for the growth of any particular species are designated the *essential metabolites* for that species. They constitute the raw materials required by the cell for both energy production and assimilation. The nature of such essential food materials is determined either by the analytic or synthetic method. The former consists first of the discovery of a medium suitable for the growth of a species by empirical means, separating the ingredients of the medium and then determining the growth-promoting property of these components, singly or in combination, with reference to that particular species. In the synthetic technique a simple basic medium of known composition is prepared and to this are added substances of known chemical structure and of likely growth-promoting value, testing at every stage the efficiency of the new product for the propagation of the species. Thus are synthetic media constructed.

The concentration of any food substance in the medium is also of practical importance. Below a certain lower limit bacteria are unable to utilise food ingredients. They are unable to grow when the concentration is high; in fact, very high concentration may actually be toxic. In adjusting the composition of culture media all these factors are kept in mind and endeavour is made to make it, as far as possible, approximate the natural food environment of bacteria.

The nutritional requirements of bacteria are extremely diverse. This is in consonance with the wide diversity of their environmental conditions. Food is necessary for energy production and for the supply of materials necessary for the synthesis of cell substance. Its source is the inorganic and organic substances occurring in nature. Bacteria in general are unable to utilise sunlight as a source of energy. Only the purple and green sulphur bacteria, by virtue of the pigment they contain, possess photosynthetic properties like the blue-green algæ and the higher plants. For the rest, forming the very vast majority of bacteria, chemical reactions form the source of energy (chemosynthetic). All the energy they need for life functions is derived from the decomposition of food substances available in their surroundings. Obviously therefore, in the metabolic scheme of

bacteria, such exothermic oxidative reactions take the place of the photosynthetic mechanism of the plant metabolism. Bacterial substance consists of carbon, oxygen, hydrogen, nitrogen, sulphur, phosphorus and inorganic salts. These have to be found in suitable forms for synthesis during growth and bacteria depend upon the environmental food substances for the manufacture of such suitable building units. The decomposition of food materials and their assimilation are both dependent on specific enzymic systems naturally associated with bacteria. The metabolism of carbon and nitrogen requires special consideration; other elements that go to make up the cell substance are readily metabolised along with them.

On the basis of their food requirements, bacteria are placed in two main classes: the *autotrophic* and the *heterotrophic*. Autotrophic bacteria are the simplest forms of bacterial life. They live by utilising carbon dioxide and ammonia as sources of carbon and nitrogen respectively. Thus they are primitive in their food requirements. From the point of view of raw food materials which they are able to utilise, these bacteria resemble plants. A few species of autotrophic bacteria contain purple and green pigments, resembling chlorophyll, whereby they are able to harness the radiant energy of sunlight for synthetic purposes. They are the purple and green sulphur bacteria (p. 38). But the majority of autotrophs are devoid of any photosynthetic mechanism, thus differing from the green plant. The chemical reactions involved in the building up of cell substance are endothermic in nature, and the energy necessary for promoting these reactions in the case of these autotrophs is derived from the oxidation of nitrogen, sulphur, iron, hydrogen and carbon, occurring either free or in the form of inorganic compounds. For example, *Nitrosomonas* and *Nitrobacter* oxidise NH_3 to $-NO_2$ and $-NO_2$ to $-NO_3$ respectively, liberating energy during the process. Energy so provided enables the reduction of the carbon dioxide to carbon which is then assimilated. Nitrogen is assimilated from inorganic sources, chiefly ammonia; it may even be from atmospheric nitrogen. The autotrophic bacteria live mostly in the soil. The group comprises only a relatively small number of species but includes some very important and interesting subgroups such as the nitrifying bacteria, sulphur bacteria and many of the iron bacteria (p. 38).

The remaining forms of bacterial life, constituting the vast majority, are lacking in simple synthetic capacity and are unable to derive their carbon and nitrogen in this way. They can live and propagate only in the presence of organic compounds of carbon and nitrogen. To this class of organisms the term heterotrophic bacteria is applied. The heterotrophs are saprophytic or parasitic and contain all the pathogenic bacteria.

It should be understood that the line of demarcation between the two physiological types, the autotrophs and the heterotrophs, is not sharp. On the borderland are certain bacterial species which exhibit both autotrophic and heterotrophic qualities (facultative autotrophs) and can live by utilising either inorganic or organic compounds according to circumstances. They are probably connecting links between the two groups.

The members of the heterotrophic group vary widely in their demand for organic food substances. These they require either for their carbon or nitrogen or for both. Some of the heterotrophs, like *Bact. coli* and *Bact. acidi lactici*, can live and multiply in a medium containing a simple carbohydrate, like glucose, as the carbon source and an inorganic salt, like ammonia, providing the necessary nitrogen. They are thus not very exacting in their food requirements. Most of the saprophytes can live in this manner. Generally, what the heterotrophs do is to break down the organic compounds into simpler intermediate products which they employ as starting materials for the building up of cell substance. For example, pyruvic acid appears to be one such important building unit formed during the carbohydrate metabolism of bacteria. The actual process of assimilation itself is still a mysterious one.

In common with the autotrophic bacteria, the majority of heterotrophs, including some of the pathogens, are able to utilise ammonia nitrogen for synthesis. Direct assimilation of amino acids may be a method of synthesis in the case of some bacteria, but generally these compounds are first disintegrated into ammonia and other similar substances. Part of the ammonia is then taken up and resynthesised into amino acids which are subsequently assimilated. Ammonia, therefore, would seem to be an essential metabolite for the vast majority of bacteria.

Some bacteria, including most of the pathogens, however, exhibit more complex food requirements. They are incapable

of utilising ammonia as the sole source of nitrogen. They are very fastidious and demand that both carbon and nitrogen should be in the form of certain preformed structural units. The organic compounds should contain particular amino acids or certain constituent groups thereof which are essential for assimilation. These organisms presumably lack the enzymic system necessary for the synthesis of such essential building units from simple materials like ammonia. Such essential metabolites, therefore, have to be included in the culture media designed for their cultivation. For example, many strains of the typhoid bacillus are not equipped with the enzymic system necessary for the synthesis of the amino acid tryptophane, although the latter is essential for their growth; tryptophane has, therefore, to be supplied preformed so as to ensure the growth of the typhoid bacillus. Such essential metabolites which cannot be synthesised by the organism itself are designated *growth factors.*

Organisms which are fastidious in their food requirements can be trained to live on simple food substances. Such adaptations can be induced by repeated subcultures on media containing falling concentrations of the complex food material and finally omitting it altogether. For example, most of the strains of *Bact. typhosum* are, on first isolation, unable to utilise ammonia as the sole source of nitrogen; they require preformed tryptophane for growth. But by growing them on media gradually impoverished of tryptophane, they gain the ability to live in the absence of tryptophane and utilise ammonia as the sole source of nitrogen.

Accessory Growth Factors. Besides the essential food materials, certain accessory growth factors have been found necessary for the growth of some bacteria. These are unable to grow in a medium properly constituted with regard to the essential food materials but bereft of such accessory factors. When the latter are added to the medium growth occurs normally. The "V" and the "X" substances (Chapter XXVI) necessary for the growth of *H. influenzæ* are examples of such accessory factors; another is the "sporogenes growth factor" for *Cl. sporogenes.* Recently, riboflavin, nicotinic acid and others have been shown to be necessary for the growth of some of the lactic acid bacilli, pimelic acid and nicotinic acid for *C. diphtheriæ* and nicotinic acid and thiamin for *Staph. aureus.* These accessory factors are organic in nature.

They are required only in minute amounts and their role in the cell economy is probably as catalysts. Apparently, the cells are devoid of the power of synthesising them. Some of these accessory factors have been proved to be identical with the various components of the vitamin B complex (p. 29). This is of interest, since it suggests a close relation between the metabolic processes of such widely divergent forms of life as bacteria on the one hand and the mammals including man on the other.

Again, certain food substances, while not necessary for growth, have been found to improve considerably the growth of many species of bacteria. Such are glucose, hæmoglobin, serum and others. Presumably, these substances either yield the essential metabolites more readily than others or provide the accessory food factors necessary for growth. From what has been said above, it will be quite obvious that the extreme diversity displayed by bacteria in regard to food requirements imposes the need for devising a large variety of culture media for their growth.

Many gaps still exist in our knowledge of the nutritional needs of bacteria. About many micro-organisms we have only incomplete data, while some have defied all our attempts to find their nutritional requirements. It has been shown above that many of the pathogenic micro-organisms require certain food factors for growth, but we know nothing about the growth demands of a few, for example the leprosy bacillus, and in consequence they have not been cultivated artificially. The higher the scale of parasitism, the more fastidious are the growth requirements and the fewer the synthetic capabilities. The growth demands of viruses illustrate this very well.

The Catabolic Activity of Bacteria—Carbohydrate Metabolism. The precise way in which bacteria deal with the various food stuffs and obtain the energy and material for synthesis, indispensable for life, is only imperfectly understood. The majority of bacteria, as alluded to above, are able to break down complex molecules of carbohydrates, proteins and fats into simpler substances. The various chemical reactions involved in the carbohydrate metabolism are generally exothermic and the energy generated is utilised to meet the demands of life.

The carbohydrates of plant origin form the best available source of energy for bacteria. They are found in nature as polysaccharides and disaccharides. The catabolism of poly- and

disaccharides, such as cellulose, starch, inulin, saccharose, lactose and maltose, consists of a preparatory hydrolysis and disintegration into monosaccharides; this is followed by oxidation of the latter into various end products. Direct oxidation of polysaccharides seldom occurs and their preliminary transformation into the constituent monosaccharides is an essential primary step. The same type of organism is not necessarily concerned in these different degradation processes. Only a limited number of organisms are capable of splitting the polysaccharides. The preliminary hydrolysis of the complex carbon molecules into simpler ones is also associated with the production of energy; but the yield from this is very small compared to that from the fermentation of monosaccharides.

The term "fermentation" is applied to the microbial disintegration of carbohydrate substances. The fermenting agents may be yeasts or bacteria, but the actual substances involved in the process are known as *ferments* or *enzymes* found in association with these microbes. Generally, the term fermentation is understood to mean the attack of carbohydrates, causing the production of acids, with or without the evolution of gas. The disintegration of protein substances, almost exclusively carried out by bacteria, is not referred to as fermentation.

The hexose sugars among the monosaccharides are readily utilised by most bacteria that are capable of decomposing carbohydrates. A wide variety of reactions, many of which are not fully understood, are involved in the process. The same bacterial species may have the capacity to cause more than one type of reaction. This is probably a provision against changing environmental conditions. During fermentation complete oxidation is a rare type of reaction, restricted to a few species; when it occurs the end products are carbon dioxide and water. Another type of reaction that may underlie fermentation is what is called the intramolecular oxidation. In this type, while one part of the complex molecule is oxidised, another part of the same molecule is reduced, resulting in the splitting of the molecule: molecular oxygen is not necessary for this reaction. In all except the first, the reactions do not proceed to completion but stop short at intermediate stages. The result is that a variety of intermediate products are formed. They vary with different bacterial species, which is of considerable differential value. There is evidence to

show that pyruvic acid is an important intermediate product. It is an easily assimilable substance and probably plays a vital role in bacterial nutrition.

Pentoses are probably decomposed on similar lines as hexoses. Arabinose, xylose and rhamnose are the common pentoses employed in fermentation reactions. A limited number of bacterial species metabolise the hexahydric alcohols, mannitol, dulcitol and sorbitol, some producing both acid and gas and others only acid. Other polyhydric alcohols often used in the fermentation reactions are glycerol and adonitol. Probably all alcohols are split up on the same lines as the hexose sugars.

As mentioned above, a variety of end products are formed during bacterial fermentation. They include acids, like lactic, formic and acetic acids, ethyl alcohol, acetone, butyl alcohol, glycerol, acetylmethyl carbinol, carbon dioxide, hydrogen and others. The nature and amount of such disintegration products depend upon the species of the fermenting bacterium and on the conditions under which fermentation occurs. The selective carbohydrate-splitting property of bacteria has long been employed as a valuable means of their classification and identification. Most of the pathogenic bacteria can break down carbohydrates to lactic, formic and acetic acids and a small amount of ethyl alcohol, with or without the production of gas which may be carbon dioxide or hydrogen or a mixture of both. The recognition of such end products in the culture tubes containing known sugars is a routine diagnostic procedure. For example, while *Bact. coli* attacks glucose, producing both acid and gas, *Bact. typhosum* or *Bact. shigæ* produces from it only acid without gas.

Protein Metabolism. Our knowledge of the protein metabolism of bacteria is more meagre than that of the carbohydrate metabolism. Particularly is this the case regarding the decomposition of native proteins. The spontaneous decomposition of dead vegetable and animal matter occurring in nature depends on the presence of bacterial life. Exclusion of the latter can be easily shown to prevent the rotting of organic matter. It is common knowledge that freezing, sterilisation by heat or the addition of disinfectants will retard or prevent putrefaction and decay. It demonstrates that without bacteria these processes cannot occur. Protein clevage occurring in nature consists of a chain of extremely complicated chemical processes. Besides,

it is not an isolated phenomenon capable of separation from the concomitant activity of diverse bacteria, other than the proteolytic ones, attacking the end products and altering them beyond recognition as the proper end products of proteolysis.

A difference is sometimes made between aerobic and anaerobic protein decomposition spontaneously occurring in nature. The term decay is used for the former and putrefaction for the latter. A superficial contrast is provided by the association of odour in putrefaction and its absence in decay; in decay, where molecular oxygen is freely available, foul smelling end products, like hydrogen sulphide, indole, skatole and mercaptans, characteristic of putrefaction, are not formed. However, the distinction is not basic and the underlying processes of decomposition are the same in both.

Relatively few bacteria are endowed with proteolytic powers; many of them are anaerobes. In them protein digestion is so marked a feature that we commonly speak of organisms as saccharolytic or proteolytic according to whether carbohydrate or protein degradation forms the predominant feature of their biochemical activities. There is no sure evidence that the proteolytic bacteria can utilise complex proteins for growth in the absence of some readily assimilable form of nitrogen. Special types of proteolytic enzymes are concerned in protein decomposition, and in the presence of protein food materials these enzymes are readily developed, initiating the process. The intimate nature of the chemical reactions involved in the enzymic hydrolysis and disintegration of complex protein molecules is very little understood. The dissimilation of the large protein molecules into smaller ones, like amino acids, constitutes, in all probability, an important preliminary step. These initial reactions probably take place extracellularly. It is only amino acids and simpler digestion products that are assimilable. Regarding the actual process of assimilation our knowledge is practically nothing.

The capacity to liquefy gelatin is regarded as evidence of the proteolytic properties in organisms. This property serves as a means of differentiating such species. With some of the organisms which liquefy gelatin, for example *Staphylococcus aureus* and *Bacterium cloacæ*, the degradation process is not carried on to the stage of amino acid production; they are, therefore, unable to utilise the liquefied product either as the source of

nitrogen or of energy. Others, like *Bacillus subtilis* and *Proteus vulgaris*, have apparently more elaborate enzymic systems; in their case, the hydrolytic process does not stop with mere liquefaction but proceeds rapidly to the formation of amino acids which they can assimilate or decompose further.

Most of the bacteria are capable of utilising amino acids. For many species these compounds are essential for growth. Though direct assimilation of certain amino acids into protoplasm is a possibility, further degradation of these compounds into simpler materials is the common course. Some of the simpler substances so formed, such as ammonia, are utilised as building stones during synthesis. This process of decomposition may also produce energy needed for the organism. As noted above, how exactly assimilation takes place is still a mystery.

The decomposition of amino acids may occur in a variety of ways, such as hydrolytic decomposition, decarboxylation, deamination and the like. Not that a single amino acid goes through all these processes during dissimilation. Also, different species of bacteria pursue different methods of decomposition. A variety of end products, such as indole, hydrogen sulphide, ammonia, carbon dioxide and others, are formed. Their kind depends upon the bacterial species causing decomposition and also, in some degree, upon the attendant conditions, like oxygen tension, pH of the medium and the like. The nature of the substances formed is also of considerable value in the differentiation of species. For example, indole, a break down product of the amino acid tryptophane, is produced by *Bact. coli* but not by *Bact. typhosum* or *Bact. shigæ*.

Bacterial Enzymes. The chemical reactions concerned in bacterial metabolism are initiated and controlled by certain substances known as *enzymes* or *ferments* which form part of the physiological make up of the cell (see above). The enzymes are elaborated by the cell from the food. A number of them are developed by the same bacterial species to which they are specific. Enzymes are essential for the metabolic activities of bacteria. The complex food materials found in the surroundings have to be broken down into simpler substances before they can penetrate into the small bacterial cell. Intracellular dissimilation and assimilation also require the interference of enzymes.

The precise chemical composition of enzymes has defied all attempts at discovery. Some of them are true secretions and

separable from the intact bacterial cell by filtration, while others can be separated only by extraction after death. By appropriate chemical processes, the former are in some cases obtainable in dry state. Whether each enzyme is a separate chemical entity has been questioned on the ground of the minuteness of space available in a cell for accommodating a multiplicity of enzymes. But only minute quantities of enzymes are necessary for action at any one time, and probably enzymes in such amounts are produced only in the presence of a substrate, so that the question of hoarding up more than a few molecules of enzymes at a time may not be a problem.

The intimate mode of action is by no means clear. The enzyme probably enters into a temporary chemical union with the molecule of the substrate on which it acts. The union causes a weakening of certain bonds, the intramolecular forces which serve to hold together the several component parts of the molecule, leading to disintegration and chemical transformation. During the process, the enzyme does not lose its individuality nor is it used up and on completion of the reaction remains undiminished in quantity, although its activity may be inhibited by any of the new products. Again, the quantity of enzyme necessary for action is disproportionately small compared to the quantity of substrate transformed by its action. Further, in some cases at least, the action of enzymes is reversible. In all these respects a close similarity exists between bacterial enzymes and chemical catalysers, such as dilute sulphuric acid, iron and others. Bacterial enzymes also behave in the same way as ferments produced in the higher animals and plants. The action of the ptyalin of saliva or of the diastase of plant origin, for instance, closely simulate the starch-splitting action of the amylase of bacteria. Although the action of enzymes is spontaneous, it is conditioned by several environmental factors. Presence of moisture, temperature and pH are some of them. Heat above 70° C. usually destroys enzymes. A temperature ranging between 35° and 45°C. is the optimum. Freezing retards their action without causing destruction. A weakly acid or alkaline reaction is the most favourable for their action, while strong acids and alkalies destroy them.

The action of enzyme is selective. A particular enzyme can act only on a particular substrate. Hence an enzyme can be

identified from the identity of the substance on which it acts. The enzymic systems acting on different groups of substances are different. Thus we have carbohydrate-splitting, proteolytic and lipolytic enzymes.

The decomposition of carbohydrates is brought about by hydrolysis; the action is specific, induced by a large variety of ferments. The conversion of polysaccharides into disaccharides occurs through the intervention of several enzymes, as many as there are types of polysacchrides. For instance, starch, cellulose and inulin are disintegrated into their respective constituent disaccharides by the ferments amylase, cellulase and inulase respectively. Specific enzymes are again concerned in the hydrolytic degradation of disaccharides into their monosaccharide components. Saccharose, maltose and lactose, for example, are transformed into their hexose constituents by invertase, maltase and lactase respectively. Any organism that forms invertase, for instance, can attack saccharose, splitting it into levulose and dextrose: an organism that develops lactase or maltase but not invertase cannot do it. Similarly, the various monosaccharide sugars are fermented through the agency of several specific enzymes. The specificity of ferment action constitutes the real basis of differences in the sugar reactions of different bacteria. When we say that the intestinal organisms are primarily classified into lactose-fermenters and non-lactose-fermenters, we really mean that the former are able to develop the ferment lactase and the latter are not.

The nature of the reaction taking place during protein decomposition is not clearly understood; it is probably one of hydrolysis. The action of the proteolytic ferments (proteases) bear a close resemblance to the action of the pancreatic ferment trypsin; a slight alkaline reaction promotes their activity. Some of the proteolytic enzymes break down the complex compounds, while others act on the simpler products. Specificity is a feature of all these reactions. The proteolytic ferments are mostly true secretions and come out of the bacterial body. Probably the large protein molecules, too large to penetrate into the small bacterial cell, are subjected to a process of preliminary hydrolysis and the resultant products of incomplete digestion are then absorbed into the cell where they are further dealt with.

The available evidence indicates that the fat-splitting enzymes also act by hydrolysis. Many bacterial species, such as *V. choleræ*, *Staph. aureus* and *Ps. pyocyanea*, possess lipolytic property.

Reaction of Media. Another important factor influencing bacterial growth is the H-ion concentration of the medium. Any given species of bacterium grows luxuriantly only within a narrow range of pH; it is the optimum pH of the species. Extending on either side of the optimum is a wider range of pH over which growth occurs but less vigorously. Outside the limits of this, growth does not occur. Extremes of pH may be inhibiting the capacity of the organism to develop the enzymes necessary for metabolism or may even be acting toxically to the bacterial protoplasm. The optimum for most of the pathogenic bacteria is a low alkaline reaction, pH 7·2–7·6; the wider range lies between pH 5·0 and 8·0. There are certain notable deviations from this. For example, *V. choleræ* grows best between pH 7·6 and 8·0, but it may grow between pH 6·4 and 9·6. The non-pathogenic bacteria usually display greater tolerance to acids or alkalies. For example, the aciduric bacilli are very tolerant to acids.

Gas Requirements. The gas requirements of bacteria vary widely. Bacteria are indifferent to the presence of free nitrogen or hydrogen. Molecular oxygen in varying amounts is necessary for the growth of the majority of micro-organisms, while a few will grow only if this gas is strictly excluded from the surrounding atmosphere. Organisms that require the presence of molecular oxygen are called *obligatory aerobes*, e.g. *B. anthracis*. Many bacteria are, however, able to grow in the presence or absence of oxygen. They are called *facultative anaerobes*. Most of the pathogenic bacteria belong to this group. When subjected to anaerobic conditions, the physiology of the facultative anaerobes tends to undergo modification. For instance, the pigment production of the chromogens is suppressed with the exclusion of molecular oxygen; spore formation does not occur in the animal tissues so long as there is restriction of the oxygen supply. Organisms that fail to grow in the presence of free oxygen are designated *obligatory anaerobes*, or simply anaerobes, e.g. *Cl. tetani*. Molecular oxygen not only inhibits their growth but is positively toxic to them (p. 31). *Micro-aerophilic organisms* are those that grow best when the oxygen present is only a trace; *C. acne* is an example of this.

Carbon dioxide plays an important role in the growth and metabolic activities of certain bacteria. *Br. abortus* will grow only under a partial carbon dioxide tension of 5–10 per cent., particularly during primary isolation. In other ways also carbon dioxide influences the metabolic activities of bacteria; for example, *Staph. aureus* elaborates its characteristic toxic products only in the presence of a higher carbon dioxide tension than usual. That carbon dioxide is an essential food material for the autotrophic bacteria has been mentioned elsewhere.

Bacterial Respiration. Most bacteria respire in the ordinary sense of the term; they take in oxygen and give out carbon dioxide as in the case of higher forms of animal life. Some bacteria, however, do not require molecular oxygen but are able to grow in the absence of this gas. Pasteur was the first to discover the phenomenon of anaerobic growth (1861). This was a great contribution to biology. At the time it was a revolutionary thought; till then life without air had been considered impossible. Now it is known that other forms of life than bacteria can grow in the absence of air.

Bacterial respiration has its counterpart in the tissue respiration of higher animals. It has a wide common ground with bacterial metabolism; that part of bacterial metabolism which is concerned with the supply of energy for the living cell constitutes bacterial respiration. Bacterial respiration, therefore, depends on specialised mechanisms essentially designed for oxidative processes.

Oxidation does not always mean addition of oxygen; removal of hydrogen from a molecule, resulting in the liberation of energy, is also oxidation. Removal of oxygen from or addition of hydrogen to a molecule is reduction. Oxidation and reduction are coupled reactions, that is, where there is oxidation there is also simultaneous reduction. A more precise definition of oxidation is removal of electrons. The addition of oxygen or withdrawal of hydrogen has the same basic effect of removing electrons just as the withdrawal of oxygen or addition of hydrogen involves an increase of electrons. In other words, oxidation, whether with the direct implication of molecular oxygen or through the removal of hydrogen, results in the release of electrons from the oxidised molecule; and the concomitant reduction results in the fixation of electrons by the reduced molecules.

A substance on oxidation loses electrons and on reduction gains electrons. In an oxidation-reduction system, therefore, there is a constant transfer or movement of electrons. Electrons in motion constitute an electric current. Its intensity can be determined by measuring the electric pressure or potential of the system. The value so obtained is also the measure of the oxidation-reduction intensity or potential of the system. The potential can be compared with that of a normal hydrogen electrode. The relative value of any system can thus be determined with reference to this standard. The oxidation-reduction potential of a system is expressed by the symbol Eh. In this way the reducing or oxidising capacity of any bacterial culture can be estimated by direct electrical measurements. It gives more accurate results than the indicator method.

In the field of biochemistry, the substrate molecule which yields oxygen is called the *oxygen donator*, and which yields hydrogen is called the *hydrogen donator*. The oxygen or hydrogen so yielded seldom escapes as gas but enters some other molecule. Such receiving molecules are referred to as *oxygen acceptors* or *hydrogen acceptors* as the case may be. Thus oxygen or hydrogen is transferred from the donator to the acceptor. This is what is meant by oxygen transport or hydrogen transport. The reactions involved in donating or accepting are under the influence of specific enzymes or catalysers; the enzymes are regarded as activating oxygen or hydrogen. Certain compounds, *e.g.* dyes, may in their reduced state donate hydrogen or accept it in the oxidised state, the reaction being reversible. That is, they act as *hydrogen carriers.*

Observations on anaerobic oxidations may be made by the Thunberg technique. In this a hydrogen carrier dye is used as an indicator of the type of reaction occurring at any time in a culture. Any dye may be used; methylene blue is convenient; it is blue in the oxidised state and colourless in the reduced state. Washed bacteria suspended in a solution of substrates, such as glucose, and methylene blue are placed in Thunberg tubes. These tubes are then evacuated and incubated at 45° C. Reduction of methylene blue, as indicated by the discharge of its colour, is regarded as evidence that hydrogen has been transported to the dye from the substrate molecule through the ntervention of dehydrogenases present in the bacterial cell. By

this technique the enzymic property of any organism can be studied. With *Bact. coli* a wide variety of substances are found to act as hydrogen donators in the presence of methylene blue. Taking as standard the reduction time of methylene blue by any given organism under certain experimental conditions, comparitive values for other systems can be obtained.

The precise way in which oxidation takes place in the bacterial cell is only very meagrely understood. Many theories have been advanced. It has been postulated that hydrogen transport is the essence of cellular oxidations. According to this theory one or more hydrogen atoms from a substrate molecule, the hydrogen donator, are activated by specific enzymes, known as dehydrogenases, and transported to some other substrate molecule which serves as hydrogen acceptor. The former molecule is thus oxidised setting free energy, while the latter is concurrently reduced in the process. Hydrogen transport is thus a mode of anaerobic oxidation and free oxygen has no place in it. It is, therefore, clear that if the cell can reduce some other substance in place of free oxygen oxidation may proceed, liberating energy and sustaining life in the complete absence of air.

In the above type of reaction two different kinds of molecules are concerned in the reaction, one acting as hydrogen donator and the other hydrogen acceptor. Anaerobic oxidation may also occur when there is only one type of substrate molecule, the decomposition occurring through oxidation of one part of the molecule simultaneously with reduction of another part. This type of anaerobic oxidation is referred to as *intramolecular oxidation*. Again, in the presence of only one type of substrate one molecule may be oxidised while another reduced. This type of reaction is called *dismutation;* it often occurs in the bacterial decomposition of carbohydrates.

As mentioned above, the hydrogen transport that occurs during respiration is conditioned by enzymic activities. These respiratory enzymes activate the hydrogen of the hydrogen donator, *i.e.* the oxidisable substrate, and enable its transfer to the hydrogen acceptor, *i.e.* the reducible substance which may be the oxygen of the air (aerobic oxidation) or some metabolite associated with the cell (anaerobic oxidation). These enzymes, consisting of a protein moiety linked with a functional prosthetic

group which is reversibly oxidised and reduced, are capable of functioning as hydrogen carriers. Hydrogen is received temporarily by the enzyme and passed on to the acceptors in an orderly manner, maintaining the continuity of the reaction. It is interesting to note that the prosthetic groups, or portions thereof, appear to be members of vitamin B complex. The dehydrogenases, the co-enzymes, the flavoprotein enzymes, the iron porphyrin enzymic system and the peroxidases all come under the category of respiratory enzymes.

Bacterial enzymes are not restricted in their scope to the activation of hydrogen and its transport. They may also induce other substances to act as hydrogen acceptors or oxygen donators. For example, *Bact. coli* has been found capable of activating nitrates under certain conditions, compelling them to part with oxygen. In the absence of air this organism is unable to grow in a medium containing only a simple carbon compound, such as lactic, formic or succinic acid, but if nitrate is added to the medium anaerobic growth will occur, the nitrate being reduced to nitrite. Nitrates in these circumstances act as oxygen donators or hydrogen acceptors. This activation is made possible by a specific enzyme possessed by *Bact. coli* in common with many other facultative anaerobes.

Hydrogen transport as a respiratory mechanism is probably well developed in the facultative anaerobes which form the vast majority of bacteria. These can also grow in the presence of air. Obviously therefore, the facultative anaerobes are provided with an alternative mechanism to function when they grow aerobically. This mechanism is the cytochrome-cytochrome oxidase system (*vide infra*). Even under aerobic conditions, the later stage of growth in a broth culture occurs under essentially anaerobic conditions as the dissolved oxygen is soon depleted in the earlier stage. But for this duplicate provision, growth would stop after the initial stage. Evidently such a dual system, therefore, imparts greater flexibility to the physiology of this group of organisms, enabling them to grow under conditions of changing environment.

It is not at present quite clear how exactly aerobic bacteria obtain their energy. The presence of molecular oxygen is an essential condition and direct oxidation of some cellular or extracellular factor forms part of the respiratory mechanism. But the exact nature of this mechanism, which involves the

utilisation of free oxygen, is not definitely understood. The theory has been advanced that hydrogen transport is the chief respiratory mechanism also in the case of aerobes. It would appear that during aerobic oxidation the hydrogen transport is accomplished through the intermediary of a respiratory pigment, *cytochrome*, which is a natural oxygen carrier present in all aerobes and facultative anaerobes.

Cytochrome is a complex system consisting of a number of related haematin compounds. When fully developed, it is composed mainly of four components: cytochromes *a*, *b*, and *c* and cytochrome oxidase or oxygenase. They are haemoproteins related to hæmoglobin. They are capable of existing in the reduced as well as oxidised states. Cytochrome *b* is auto-oxidisable but not *a* and *c*. The latter two, however, are readily oxidised by cytochrome oxidase which in its reduced form is readily auto-oxidisable by atmospheric oxygen. The full complement of cytochrome components is present in some bacterial species, e.g. *B. subtilis;* only some of them are present in others, e.g. *Bact. coli;* and none in the obligate anaerobes.

The cytochrome system serves as hydrogen carrier in the same way as described in the case of dyes and natural catalysers. It receives hydrogen from intracellular substrate donators, which have been specifically activated by the bacterial dehydrogenases, and delivers it to the hydrogen acceptor which is the molecular oxygen of the air. In so doing the cytochrome itself undergoes alternate reduction and oxidation. The oxygen of air, according to this theory, functions only to maintain the oxidised state of the cytochrome carrier and not to directly oxidise the substrate. The oxidation of cytochrome is brought about through the agency of specific oxidases. Thus cytochrome acts as an intermediary between dehydrogenases, activating the hydrogen of the substrate on the one hand, and oxidases, activating molecular oxygen on the other. Certain cytochrome derivatives may also act as direct catalysts, promoting oxidation of substrates without the intervention of specific dehydrogenases.

We have as yet but scanty information regarding the respiratory mechanisms of the anaerobic bacteria. Available evidence indicates that in their case also the mechanism of oxidation is hydrogen transport. These organisms lack the respiratory pigment, cytochrome. Whether any other type of carrier is present

or not is unknown. Certain peculiarities regarding their respiratory metabolism have, however, been brought to light. Paired amino acids are essential for their growth; not only the hydrogen donator but also the hydrogen acceptor should be an amino acid. These oxidation-reduction reactions are under the control of specific enzymes. It is also imperative that the oxidation-reduction potential (Eh) of the system should never rise above a certain low limit. Why a higher reducing potential or intensity of the medium should produce the above adverse effect on their growth is not clear (*vide infra*). By suitable mechanisms, about which we have very little knowledge at present, the Eh is always maintained sufficiently low. Sulphhydryl compounds, such as glutathione and cysteine, are able to keep down the Eh in culture media in the presence of traces of copper.

The reason why atmospheric oxygen is toxic to anaerobic bacteria is obscure. One of the explanations given is that in the presence of air these organisms develop hydrogen peroxide during growth which is toxic to the cell. Many other bacteria also form hydrogen peroxide during growth; but, whereas they also develop a catalase, *peroxidase*, which would rapidly destroy the peroxide as it forms preventing accumulation to toxic concentrations, the obligate anaerobes, which are peroxide sensitive, lack the capacity for developing the protective catalase, thus exposing the cell to the toxic action of hydrogen peroxide. This still remains a theory and no substantial evidence in support of it has been so far brought forward.

Temperature. Each species has a definite range of temperature over which growth is not interfered with. The limits of this range are called the minimum and the maximum temperatures for that species. This range varies with different species, and for most pathogens it lies between 15° and 45° C. Between the minimum and the maximum there is a narrower range, the optimum temperature, within which the species thrives well. The optimum temperature for any species is that of its natural habitat: in the case of human pathogens it is round about 37° C. Below the minimum temperature bacteria are not necessarily killed, though their activity may be curtailed; but when exposed to a higher temperature than the maximum, death ensues more or less quickly.

Bacteria are ramarkably resistant to low temperatures. The optimum temperature of the parasitic bacteria of cold-blooded

animals is necessarily low. Even some of the human pathogens can resist extremely low temperatures for short periods; for example, about 10 per cent. of the typhoid bacilli in a culture have been found to live after thirty minutes' exposure to −175° C. Spores are scarcely affected by such low temperatures (p. 139).

Temperatures slightly above the maximum may not kill an organism if exposed to only for short periods; they may, however, interfere with the normal functions, such as spore formation, virulence and the like. But if exposed to such temperatures for long or exposed to still higher temperatures for any length of time bacteria are rapidly destroyed. Non-sporing pathogens ordinarily perish rapidly above 45° C. Spores withstand high temperatures for a much longer time than the vegetative forms. Some species of saprophytic bacteria grow best between 50° and 55° C. with a maximum limit of 70° or even 75° C. for viability. They are called *thermophilic bacteria;* their usual habitat is the soil.

Heat kills bacteria by coagulating the proteins. This is not a sudden process but takes some time for completion at any particular temperature above the maximum for growth. That is, the killing is also a function of time. For instance, the tubercle bacillus is killed in thirty minutes at 58° C., twenty minutes at 59° C., and in two minutes at 65° C. In a culture a few organisms are always more resistant than the rest and they survive to the last. The lowest temperature, above the maximum for growth, at which a particular organism is killed in a certain definite time is called the *thermal death point* of that organism. Each species has its own thermal death point which is also influenced by other factors as pH, number of organisms and age of the culture. That the thermal death point of the pneumococcus is 52° C. means that a culture of this organism will be completely sterilised at 52° C. in a given time—10 minutes; but if exposed for less than 10 minutes varying number of them will survive, depending upon the time of exposure.

Micro-organisms withstand dry heat longer than moist heat. Vegetative forms are destroyed in a few minutes by moist heat at 100° C., whereas to produce the same effect a temperature of 120°–130° C. dry heat maintained for one and half hours is necessary. Spores are extraordinarily resistant to any form of heat. In this respect spores of different species vary widely;

for example, tetanus spores resist boiling for 15–90 minutes, whereas anthrax spores are killed in 10 minutes (see Chapter VII).

Moisture. More than four-fifths by weight of the bacterial body consist of water. Hence moisture is essential for growth; deprivation of it is harmful to life. Some organisms are said to withstand drying for long periods, *e.g.* the tubercle bacillus. But it may be that the amount of moisture required by the micro-organisms for the maintenance of life is extremely small. Spores are far more resistant to desiccation than the vegetative forms; anthrax spores remain viable for years under such conditions. The rapid withdrawal of moisture at low temperatures, however, is not injurious. Cultures keep alive for several years if they are rapidly dried and preserved *in vacuo* and at low temperatures and in the dark (p. 137).

Light. Direct sunlight is injurious to the growth of Bacteria. Most bacteria are destroyed if sufficiently exposed to light. The destructive agency is the ultraviolet content of the rays. Growth occurs best in the dark.

Chemicals. Many chemical substances are protoplasmic poisons. The presence of any in the culture medium either inhibits growth or destroys the organism, depending upon the concentration of the chemical substance. More detailed reference to this subject is made in the chapter on the destruction of bacteria.

Mutual Influences. The study of the mutual reaction between microbes and their environment constitutes ecology. Every form of life, from the lowest to the highest, is subject to influences from its surroundings. Such influences emanate either from other similar or dissimilar forms of life, inanimate objects or from physical forces. Bacteria are particularly sensitive to such environmental forces, reacting readily and profoundly to their impact. In nature, micro-organisms live together in mixed cultures and a pure culture is an exception. In this association they are not always passive with respect to each other but are often subject to favourable or adverse influences from neighbours.

When different species of bacteria exist together, some may exert a favourable effect on the growth of others, *i.e. symbiosis* or some may be antagonistic to the growth of others, *i.e antibiosis* or antagonism (p. 191). The beneficial effect of one

species may be due to the production of favourable conditions for the growth of another. For example, the staphylococcus synthesises the "V" substance necessary for the growth of the influenza bacillus and the latter, therefore, grows luxuriantly in the vicinity of staphylococcal colonies; the aerobic organisms in an open wound use up the oxygen in the immediate surroundings and create the gas phase compatible with the growth of anaerobes. The antagonistic effect may be due either to the depletion of the surroundings of all suitable pabulum or to the formation of injurious substances, like acids, alkalies, hydrogen peroxide or toxins, by the inhibiting species. Examples are acid inhibition of the proteolytic flora by the lactic acid bacillus in the large intestine, inhibition of growth of diphtheria and other organisms by the pyocyaneus bacillus and the action of *Penicillium notatum* on the growth of the Gram-positive and some Gram-negative bacteria.

The Products of Bacterial Growth. In addition to the assimilation of protoplasm, bacteria synthesise certain other constituents from the food substances available in their neighbourhood. Most of these have been discussed previously. Such are the capsular polysaccharide material which confers immunological specificity upon the bacterial species, metachromatic or volutin granules which are of some practical importance in diagnosis and the various enzymes which are essential for the physiological activities of bacteria. About the formation of hydrogen peroxide by most bacteria mention has been made above. The synthesis of substances like hæmolysin has been dealt with under toxins. Two other chemical products remain to be discussed, *viz.* bacterial pigments and bacterial toxins; the latter, from the point of view of medical bacteriology, are the most important of all the products elaborated by bacteria.

Chromogenesis. Not all bacteria are pigment producers. Many species, however, develop during growth characteristic pigments, e.g. *Ps. pyocyanea*, *Staph. aureus*, *Chr. prodigiosum* and others. Such organisms are known as *chromogenic* bacteria. A single species may elaborate more than one pigment; for instance, *Ps. pyocyanea* synthesises pyocyanin and fluorescin. The pigments are stored round the bacterial cell in the form of granules or they diffuse out of the cell, colouring the surrounding medium. From red to violet almost every colour is seen.

The chemical composition of pigment is in many cases not known. The structure of pyocyanin has been worked out; it is a phenazine derivative. Many of the bacterial pigments are soluble in the fat solvents, alcohol, acetone, ether and chloroform, but insoluble in water (lipochromes). Only a few, like fluorescin, are soluble in water; they are not soluble in ether, alcohol or chloroform and are probably related to anthocyanins. What function these pigments discharge in the physiology of the cell is not clear. They have no part in the pathogenesis. Cytochrome pigments, a lot by themselves, appear to be related to bacterial respiration. Their presence does not impart any tint to the bacterial colonies. Pyocyanin has been shown to increase the respiratory activity of the cell. The pigments of the sulphur bacteria have photosynthetic capacity and are connected with bacterial nutrition.

The value of pigments to the laboratory worker lies in the help that they give in the identification of species; they have also some classificatory value. But it should be remembered that pigment production is an unstable character, dependent upon various circumstances, such as the nature of the medium, oxygen tension, temperature and continued cultivation. Pigment production is particularly favoured by carbohydrates like the potato or starch medium. Oxygen is necessary for the formation of pigments. The colour production of *Ps. pyocyanea* is suppressed when grown anaerobically. Ordinary temperature (20°–28° C.) appears to favour a richer colour production than higher temperatures. For example, *Chr. prodigiosum*, which develops a brilliant red colour at ordinary temperature, fails to develop it when grown at 37° C. With few exceptions, pigment production occurs best in the dark. Certain mineral salts are necessary for the elaboration of some pigments, for example, phosphate and sulphate for fluorescin.

Bacterial Toxins. These are products of bacterial activity and constitute the actual agents in the pathogenic processes involved in bacterial infection. They are divided into two classes—the *exotoxins* and the *endotoxins*. The exotoxins are those which readily diffuse out of the bacterial cell into the culture medium. Hence they are also termed *extracellular toxins* or *soluble toxins*. After removal of the organism from the cultures in which they grow, the filtrates are toxic. Therefore, they are

also sometimes spoken of as *filterable toxins*. The exotoxins have not been isolated in a pure state and their chemical composition is not known. Probably they are protein in nature. They are some of the most potent poisons known. Some of the pathogenic organisms produce powerful extracellular toxins, *e.g. C. diphtheriæ*, *Cl. tetani*, *Cl. botulinum*, *Staph. aureus* and *Str. pyogenes*. Most of the pathogenic bacteria, like *V. choleræ*, *Bact. typhosum* and *N. gonorrhoeæ*, do not develop any toxins separable from the bacterial body. Consequently their cultures after removal of the bacterial cells are non-toxic. In their case the dead and the disintegrated bodies constitute the toxic material. Hence endotoxins or intracellular toxins. Whether these bacteria synthesise any separate toxic material which remains intimately mixed up with their protoplasm is doubtful. For detailed discussion refer Chapter IX.

Besides the exotoxins and the endotoxins, there are several other bacterial products with special effects, which are also concerned in infection and its subsequent course. In this category may be included certain substances resembling exotoxins like hæmolysin and leucocidin, bacterial enzymes like fibrinolysin and coagulase, bacterial substances like the "spreading factor" which are probably the labile antigens, haptens or partial antigens including aggressins, and bacterial allergens.

Heat and Light. Large amounts of energy are generated by bacteria during growth. Only a portion of it is consumed during the performance of life functions. The rest is liberated as heat. The production of the latter is well illustrated by the relatively high temperature developed during active bacterial decomposition occurring, for example, in manure heaps and damp hay. Production of light is also associated with many species of bacteria, for example the photogenic bacteria commonly found in sea water.

Bacteria capable of emitting light are widely distributed in nature, but more commonly in sea water. They are often found on decomposing fish and meat. A large number of species have been described. All the phosphorescent bacteria are aerobic. Although phosphorescence is a natural property of their living protoplasm and is not the result of oxidation of any photogenic substance emitted by them, yet the phenomenon occurs only in the presence of oxygen. The photogenic organisms

can live without emitting light; with death they lose this property. The significance of this property to the micro-organism is unknown.

Nitrogen Fixation. The ability to use nitrogen of the air for assimilation is an attribute of certain species of bacteria widely distributed in nature, but particularly in the soil. Soil is constantly losing its nitrogen supply through utilisation of it by countless plants. This would lead to complete depletion were it not for the replenishing processes continually taking place in nature. These processes are mainly dependent on bacteria for their fulfilment.

The keeping up of the nitrogen supply of the soil is effected in two ways: one by the return of the nitrogen consumed by the plants and the other by the fixation of atmospheric nitrogen. Plants obtain their nitrogen by absorption of simple salts (NO_3) from the soil. These are synthesised as plant substance. Plants form the food of animals and thus part of the nitrogen consumed from the soil by plants reach animals. Bacterial decomposition of dead animal and plant bodies and animal excretions and secretions, therefore, eventually lead to the return of the nitrogen consumed by plants to the soil.

Enrichment of soil with atmospheric nitrogen had been known for a long time. But its biological basis was brought to light only comparatively recently. Now it is known that a limited number of bacterial species are able to fix the free nitrogen from air and make it available as food for plants. Two types of bacteria are involved in the process: certain free-living bacteria and bacteria living symbiotically with leguminous plants. The former comprise the important nitrogen-fixing organism *Cl. pastorianum*, which is a spore-bearing obligatory anaerobe, and the aerobic *Azotobacter* group. These organisms have a universal distribution in soil. It is not known how exactly they fix molecular nitrogen. Nor do we know in what usable form the plant gets it. What all intermediate products are formed in the course of assimilation also remains equally obscure.

The root nodules of leguminous plants are colonies of bacteria of the *Rhizobium* group growing in large root cells. These organisms also occur free in the soil, but only when they are in intimate association with the host plants do they fix nitrogen and not otherwise. Root nodules also occur in plants other

than the *Leguminosæ*, but no nitrogen-fixing role has been proved in such cases. The occurrence of leaf nodules containing nitrogen-fixing bacteria has also been reported.

The Nitrifying Bacteria. The nitrifying bacteria constitute an important group of autotrophs present in the soil and concerned vitally with soil fertility. They derive their energy by oxidising ammonia to nitrates. The transformation occurs in two stages and is brought about by two entirely different types of nitrifying bacteria. *Nitrosomonas* and *Nitrosococcus* convert ammonia to nitrates and the next stage of oxidation of nitrites to nitrates is continued by *Nitrobactor*. These processes are very important in that it is in the form of nitrates that plants obtain their nitrogen from the soil. The reverse process of reducing nitrates to ammonia and even to free nitrogen also occurs in the soil. Organisms which bring about this change are called denitrifying bacteria. The *Chromobacteria*, many spore-forming aerobic bacteria and the colon-typhoid group all display this property.

The Sulphur Bacteria. The sulphur bacteria or thiobacteria consist of two groups: the coloured and the colourless. The former comprises the purple and green micro-organisms. They contain two pigments, red and green, which make possible a photosynthetic mechanism analogous to that of the green plant. The colourless ones are some of them long and filamentous, showing close relationship with lower fungi. The sulphur bacteria derive their energy from the oxidation of sulphur and its compounds. They are widely distributed in nature and are found in the water of sulphur springs.

CHAPTER III

THE MICROSCOPE

In the study of micro-organisms, we are dealing with forms of life far beyond the perception of the unaided eye. Small wonder then that the advance of bacteriology, an observational science, had to keep pace with the development of the microscope. Various magnifying instruments are of supreme interest and importance to the bacteriologist. As lenses constitute the vital part of optical instruments, their discovery obviously was an essential prerequisite in the development and perfection of the microscope. The invention of lens was one of the greatest events in the history of science. Its first use was, in all probability, in the making of spectacles, a simple binocular magnifying device. When the latter was first discovered, history does not tell. Who devised it first, whether it took shape from the ingenuity of a craftsman taught by experience and driven by necessity or from the searching curiosity of some ancient and unknown philosopher are alike hidden in the mist of unrecorded antiquity. What is known with certainty is that reading glasses were in use in Europe as early as 1280 A.C.

Antony Van Leeuwenhoek, a Duch maker of lenses, was the first to make any real attempt at microscopical observation and study of micro-organisms (1676). He began this with the aid of a simple magnifying lens mounted on crude support. His untiring work opened the way to a vast world till then unknown. His careful examination of various materials, like saliva, infusions, water and others, resulted in the discovery of bacteria and protozoa and so he is rightly hailed as the father of microbiology. Undoubtedly he was the forerunner of men like Pasteur and Koch. It may also be mentioned here that it was he who first discovered the red corpuscles.

The invention of the compound microscope antedated Leeuwenhoek's discovery of microbes by nearly a century. In 1590, Hans Jansen and his son Zaccharias of Middleburg in

Holland made the first compound microscope. For a long time thereafter, this discovery was not utilised for the study of microscopic forms of life nor was it rapidly improved upon by further work. It is the last hundred years that have brought the compound microscope to such a high pitch of excellence as we have it today. Remarkable improvements have been made within recent years. Fineness and precision inconceivable to the ordinary mind have been obtained. Convenience and comfort in work have been greatly enhanced. A high degree of magnification consistent with resolution and clearness has been made possible. The introduction of immersion lenses, the substage condenser and the apochromatic lenses are some of the notable landmarks. Many modifications of the compound microscope have been designed in order to meet special needs. They are the sterioscopic microscope, the polarizing microscope and the metallurgical microscope. The optical principles involved in the construction of these variants, however, remain the same.

Compound Microscope. The compound microscope consists of the *magnifying* (image forming), the *illuminating* and the *mechanical* components, all suitably supported on a stable *stand*. The object glass and the eyepiece mounted on the tube constitute the magnifying part, while the condenser and the mirror together form the illuminating part. The eyepiece, the object glass and the condenser together are known as the *optical parts* of a microscope.

The stand is made of solid iron so that it may support the load it has to carry and at the same time impart balance and stability to the instrument. It is so designed as to support the various optical parts with perfect alignment. The stand consists of a vertical *pillar* supported on an elliptical or horse-shoe shaped *base* or *foot*. Hinged at the upper end of the pillar is the *limb* or *arm* of the microscope, which carries the illuminating apparatus, the stage and the observation tube. It is made of a single curved piece of iron well fitted to serve also as a handle.

The limb may be inclined about the hinge at any angle, but this necessity is obviated in the modern stands by the provision of inclined tubes. Though it is more expensive, the inclined tube is decidedly more advantageous as it keeps the stage horizontal while at the same time gives greater comfort

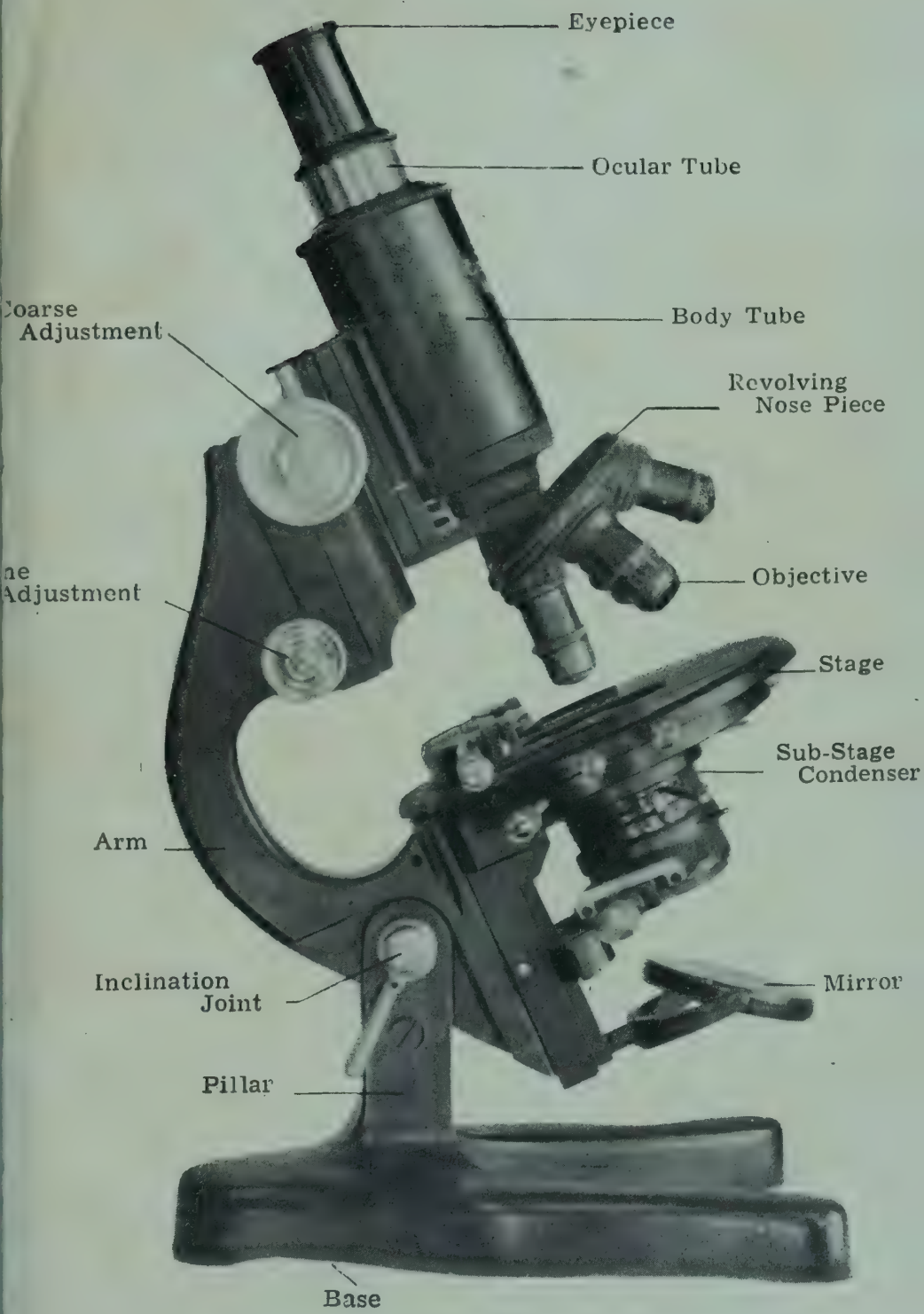


FIG. 3. The Compound Microscope

during work. If the stage is inclined, as it happens when the body is inclined at the hinge in the common microscope with the straight body, the oil will slop over the slide on the stage when using oil immersion lens; it is also disadvantageous when dealing with fluid preparations. In the inclined tube the path of the light rays is deflected by the interposition of a prism at the incline.

Included under the mechanical parts are the body tube with its coarse and fine adjustments, the inclination joint, the mechanical stage, the focusing adjustment of the condenser and the horse-shoe base. The *body tube* is a wide tube attached to the upper end of the limb; telescoped into the upper end of it is a narrower graduated tube designated the *draw tube*. The body tube is worked up and down by two separate arrangements, one the *coarse adjustment*, by means of the rack-and-pinion mechanism, for coarse movements and the other, the *fine adjustment*, worked either by means of gear arrangements or by the micrometer screw device, for delicate focusing movements. The position of the slow motion varies with different makes of the microscope. In the older ones it was located at the top of the limb and worked vertically up and down, but later models have it located at the upper end of the limb parallel with the coarse adjustment. Some of the more modern ones have the fine adjustment near the lower end of the limb either coaxial with the hinge pin or above the foot. The graduated drum on the focusing head of the fine adjustment registers the value of the fine movement of the observation tube. It is graduated in 100 divisions, each of which represents a movement up or down of the tube of 0·001 millimetre. One complete revolution of the milled head, therefore, raises or lowers the tube by 0·1 millimetre. The range of excursion of the fine adjustment is very narrow, two to four millimetres. It is limited by means of a stop and in many makes the outside limits are shown by two marks on the handle. There is a single mark on the tube mount which shows the position of the tube in relation to the two marks on the handle. Both the motion heads are provided with safety ratchets to prevent any forcible turning beyond the stops.

Attached at the lower end of the limb is a platform called the *stage* which serves to support the specimen under observation. The stage is either rectangular or circular in shape with

a central aperture to allow the light rays to pass up. The simplest type is the fixed stage with a diameter or side of 12 centimetres. The slide on which the specimen is mounted is kept in position by means of a pair of spring clips attached to the upper surface of the stage and movements of it in the horizontal plane are executed by the hand. Steady and uniform movements, however, are much better executed by means of a *mechanical stage* which may take the place of the fixed stage. Mechanical stages are of the simple or the large design. The simple mechanical stage can be revolved about the optical axis or moved within a limited range in the transverse plane by means of screws. The large compound mechanical stage, also called the *built-in mechanical stage*, allows very much wider range of movements. *Attachable mechanical stages* can be fitted on to the simple fixed stage, thereby imparting to the latter all the advantages of a compound movable stage. Mechanical stages are often graduated. They are very useful in relocating an object in the field by noting its previous position read by the verniers. The stage is not capable of movement in the direction of the optical axis except in certain special designs not used in biological sciences.

Below the stage and attached to the lower end of the arm is the *substage*. It is worked up and down by means of a rack and pinion or a spiral screw. The substage carries a collar which receives the *substage condenser;* this concentrates the light rays reflected by the mirror to the object plane in the optical axis. It is provided with an *iris diaphragm* to regulate the amount of light entering the condenser. The provision for movement of the condenser along the optical axis meets the contingency that all slides are not exactly of the same thickness; the condenser can be moved up or down and focused to the exact point of the object, producing the optimum results. By the provision of centering screws, the condenser can be properly centered and this is very important when using objectives of superior optical qualities. Commonly the two-lens *Abbe type* of condenser is the one used; but when apochromatic or semi-apochromatic objectives are used, a condenser of the same optical property should be used. On swinging out the *diaphram carrier*, which is provided just below the condenser mount, the condenser may be removed from its sliding sleeve and replaced by a

different type of condenser such as a swing-out condenser, a dark field condenser or some other illuminating device as need be. About 10 cm. below the stage, a plano-concave *mirror* is mounted on a gimbal fitting, so that it can be turned in any direction for reflecting light, which is attached to the lower end of the *tail piece*. The plane mirror is used when there is a condenser and the concave mirror in the absence of a condenser. The concave mirror acts also as a substitute for the condenser.

Attached to the lower end of the body tube is a revolving device called the *nosepiece*. It is provided with one or more sockets to receive as many objectives of different magnifying powers. The advantage of the revolving nosepiece is that it enables to rotate into position the desired objective without the necessity of unscrewing one objective from the tube and screwing another one. The draw tube, which is graduated and usually extensible, holds the eyepiece at its upper end. It has often a diaphragm provided at its lower end to cut off any reflections from the inner wall of the tube. In some microscopes the tube is non-extensible and single, permanently set at its standard working length.

Primary magnification is effected by a system of lenses suitably mounted and functioning as a single convex lens with an equivalent focus. It is immediately above the object and is called the *object glass* or simply the *objective*. It forms a magnified image. The lens of this series nearest to the object is called the *front lens*. The objectives are screwed on to the revolving nosepiece. The lengths of the objectives are so adjusted that when they are rotated on the nosepiece into position they are approximately in focus, *i.e.*, they are parfocal. A second mounted system of lenses, called the *eyepiece* or *ocular*, is slided down into the draw tube at its upper end. The lower lens of the eyepiece system is called the *field lens* and the one nearest to the eye is called the *eye lens*. The eyepiece magnifies further the magnified image formed by the objective.

The binocular microscope is a great improvement. It permits the use of both eyes and so reduces the eyestrain considerably. With a little experience the capacity to blend perfectly the two images from the right and left oculars may soon be achieved. When it fails with binocular microscopes with parallel ocular tubes, as it does with some people, those with converging ocular

tubes will be found satisfactory. The modern stands are substantially constructed so that the monocular and binocular bodies can be interchanged. Directly above the objective a compound prism is interposed in the course of the light rays. It divides the rays equally into two halves and deflects them to the right and to the left. Simple prisms, arranged one on either side of the central prism, receive the half light and direct it up to the eyepieces of the microscope. It is important to remember that binocular microscopes require double the amount of light needed by the monocular ones, and so when working with the former a sufficiently powerful source of light should be employed. The eyepiece tubes are capable of lateral movement and by means of a milled head arrangement provided between them the interocular distance can be adjusted to suit individual observers. In order to meet any differences in the focus of the eyes of the same person, one of the ocular tubes is constructed of adjustable length so that it can be extended or shortened as need be. Inclined binocular bodies are a feature of some of the more recent makes.

The body of the microscope has usually a length of 140 mm., but this distance can be increased up to 200 mm. or more by sliding up the draw tube. The draw tube scale may read from the lower end of the body tube and does not include the thickness of the nosepiece which is usually 15 millimetres. The *optical tube length* of a microscope is the distance between the back focal plane of the objective and the front focal plane of the eyepiece. The front focal plane of the eyepiece is arranged to coincide with the plane at which the image would be formed by the objective if the field lens did not intervene. The *mechanical tube length* is the distance between the upper end of the draw tube to the lower edge of the revolving nosepiece. In most makes of microscope it is 160 mm. and their objectives are corrected to this distance. But the Leitz objectives are corrected to a tube length of 170 millimetres. When the draw tube scale does not include the thickness of the nosepiece, allowance must be made for this when adjusting the tube length. For instance, if the objective is designed for 160 mm. tube length, the draw tube is set at 145 mm. which, together with the thickness of the nosepiece, make the required 160 millimetres. The correct tube length is generally indicated on the draw tube by an engraved

ring. It is essential for good results that in all manipulations of the microscope, particularly when apochromatic objectives are used, the length for the tube should be correctly adjusted by drawing out the draw tube to the required extent.

Objectives and Oculars. Objectives are designated by their focal lengths and oculars by their factorial magnification. The focal length, the magnification and the numerical aperture of the objectives are all generally inscribed on the objective mount. The objectives in common use are the 16 mm., or $\frac{2}{3}$ inch, 4 mm., or $\frac{1}{6}$ inch, and 2 mm., or 1/12 inch. Of these, the former two are called the dry lenses and should never be used with oil. The 2 mm. objective is an immersion lens. Immersion lenses, as they are designed to work with a wide cone of light, should always be used with a suitable immersion fluid. Otherwise, it is impossible to obtain the best results of which they are capable. Usually cedar wood oil is used as the immersion fluid; hence the name *oil immersion lens*. Brightness and resolution depend upon the amount of light admitted through the aperture of the front lens. Rays of light from any point on the object would undergo refraction as they emerge out of the cover glass into the rarer medium, air, with the result that the circumferential rays would be deviated away from the aperture and so only a portion of the rays from the point would enter the front lens. If, however, cedar wood oil, a medium possessing the same refractive index as glass (1·515), is interposed between the cover glass and the objective, the loss of rays due to refraction can be avoided by establishing continuity of optical texture. Brightness and resolution are thereby very much improved. That is the rationale of using immersion fluid. Other immersion lenses rarely used are 3·5 mm., or 1/7 inch, and 1·5 mm., or 1/16 inch.

The *correction mount* often provided in the high power objective is a device whereby the distance of the back lens from the other lenses of the objective can be varied so as to adjust the objective for different thicknesses of the cover glass by simply turning the correction collar.

Eyepieces employed for ordinary work are of the Huyghenian type. The Huyghenian eyepiece consists of two plano-convex lenses, one large and one small, with a diaphragm between them. The convex surfaces of both lenses face downwards towards the objective. It is so designed as to combine the qualities of high

power and large light collecting capacity. Its equivalent power is nearly as great as the power of the smaller lens and its light gathering capacity is much greater than that of the larger lens. The eyepieces are graded according to their magnification from 2 to 50 and designated by these numbers which are engraved on their mounts. The higher ones of the series, above 10 ×, are not commonly used as they cause distortion and haziness of the image. There are various other types of oculars such as the compensating, the Ramsden and so on.

The optical parts of the microscope are also classified according to the degree of correction effected in their inherent defects, the spherical and the chromatic aberrations. Spherical aberration is caused by the difference in the refractive powers of the peripheral and the central portions of a lens. The peripheral rays are refracted to a greater degree than the central, while the axial rays are not refracted at all but transmitted along the optical axis. The result is that all the rays from a point are not brought to the same focus. The marginal rays, as they are refracted more than the central, are focused on the axis at a point nearer to the lens than are the more central rays. A point, therefore, instead of appearing as a bigger point will appear as a distorted one. Chromatic aberration is due to the composite nature of white light and the lens splitting it up into its component colours. These component colours are not refracted to the same extent; for example, the blue rays are more refrangible than the red; consequently they are brought to a focus nearer to the lens than the red. As a result, fusion of the various colours necessary for the formation of white light does not occur. Consequently the image is fringed with colours, rendering the outline hazy. Lenses corrected for spherical aberration are known as *aplanatic* lenses. The same method of correction is applicable as in achromatism, *i.e.* by the combination of positive and negative lenses of different powers. Those corrected for chromatic defects are called either *achromatic*, *fluorite* or *apochromatic* lenses according to the degree of correction.

The amount of colour correction can be varied by varying any one or all the following factors: the number of lenses used in the combination, their interrelationship and their texture. The achromatic objective is a composite system consisting of

positive lenses of crown glass, having low dispersive power, and negative lenses of flint glass, possessing very high dispersive qualities. Obviously this combination also corrects the spherical aberration. This method seeks to combine two of the spectrum colours and a fair degree of correction has been obtained. They are quite good for ordinary purposes. With the oil immersion achromats it is usually enough to use the Huyghenian oculars, but better results are undoubtedly obtained with the compensating eyepieces. The apochromatic objectives are made of fluorite and there are ten or more lenses in their formation. This mineral is transparent, has a refractive index lower than all other existing optical minerals and possesses very low dispersive power. In the design of the apochromat, it is sought to synthesise not only the dominant colours but also the intermediate ones and indeed a very high degree of correction has been achieved, leaving unfused only the residual colours. Maximum resolution is obtained by the use of the apochromats, making a very high degree of effective or useful magnification possible. They are used only for critical work, such as microphotography, and then only in combination with compensating eyepieces. When apochromats are used, special care should be taken also to adjust the tube length accurately and to employ a properly centered condenser of the same optical qualities. The mineral fluorite is very costly; consequently the apochromatic lenses are very expensive. The fluorite or semiapochromatic objectives occupy an intermediate position between the achromats and the apochromats as regards colour correction. Fluorite objectives, with the same number of lens elements in their system as achromatic objectives, are far better corrected than the latter because of the use of fluorite in their make.

The source of light may be sunlight or any artificial light from a microscope lamp. The light that comes from white clouds or a blue sky is usually considered the best form of sunlight for microscope illumination. The uncertainty of sunlight, particularly in the higher latitudes, is a great drawback. Artificial light, on the other hand, is a constant source and can be easily controlled. The artificial source must be provided with a mechanism (an iris diaphragm) to regulate the amount of incident rays. The illumination should be just sufficient to fill with light the back lens of the objective. More than this will

cloud the image. Many types of microscope lamps are available from the simple substage lamp, which may be used in place of the mirror, to the very powerful and elaborate lamp equipped with condensing lens, reflector and iris diaphragm.

Rays of light incident on the mirror are reflected along the optical axis and passed up to the condenser. The amount of light admitted to the condenser is regulated, if necessary, by the iris diaphragm provided below the condenser. The two-lens Abbe condenser is the one commonly used. Its position has been arranged such that the plane of the object practically coincides with its focal plane. The condenser, therefore, forms a very bright image of the source of light in the object plane at the optical axis, illuminating the object brilliantly (*critical illumination*). When racked up, the surface of the condenser is not quite flush with the surface of the stage but is about 0·1 mm. below it. The *focal intercept* of the condenser is the distance between the surface of the condenser and its principal focus. This distance determines the thickness of the glass slide employed to mount the object. The distance between the upper surface of the cover glass and the free surface of the front lens is called the *working distance*. Part of the rays travelling up from any point on the object enters through the front lens in a cone-shaped manner. The angular size of the cone of light entering through the front lens from a point is called the *aperture*. Were it not for the field lens of the eyepiece the object glass would form a real, enlarged, inverted image of the object at the plane of its conjugate focus which lies between the field lens and the eye lens of the eyepiece. But as the field lens intervenes in the course of the rays before forming the real image, these rays are deflected and the resultant image formed by the objective and the field lens together lies at a plane a little lower than this. It is this image that is viewed by the observer through the eye lens. Its position coincides with the front focal plane of the eye lens. The rays passing through various points of this image pass up and emerge out of the eye lens and through a small circular space and the eye, when placed at this point, receives these rays. To the unaccommodated eye they would appear to be emanating from an image considerably enlarged and situated far away below the microscope. But this distance to the accommodated eye, when it sees clearly, must be the same as the distance of distinct vision, *i.e.* 25 cm.,

and the apparent or virtual image formed by the combination of lenses constituting the image forming apparatus of the microscope would seem to be at this distance. Thus, the primary magnified image is again magnified by the ocular, causing finally a double magnification. As stated above, the pencil of rays appearing to proceed from the virtual image are condensed above the ocular at the plane of the back principal focus of the entire microscope. It is also at this plane where the image of the aperture stop of the objective is formed by the eyepiece. It appears as a bright disc above the eyepiece and is variously known as the *Ramsden circle*, *eye point*, *exit pupil*. This circular area is very small, enabling the narrow pupil of the eye placed at that plane to receive a large bundle of rays. Retina takes up these emergent rays and perception of the object in a very much magnified form results. To get a full image formed by the microscope the eye should be placed exactly at the eye point which is about three to four millimetres above the upper surface of the eye lens.

The magnified image thus formed should be bright, well defined and capable of revealing minute structural details (resolution), exposing as much of the deeper structures as possible (depth of focus). Flatness of field is yet another point aimed at. These are the other important requirements to be satisfied by the magnifying components of a good microscope.

Magnification. With ordinary light a microscope can in theory be made to magnify objects indefinitely, but beyond a certain limit magnification is rendered useless as the image becomes blurred. The magnification that can be obtained without sacrificing the other important qualities of the image is called the *effective magnification*. It is this effective magnification that is sought to obtain through the agency of a microscope and not its possible magnification.

Total magnification is the product of the initial magnification due to the objective and the secondary magnification due to the ocular. The initial magnification should be first determined. It may be recalled that the objective functions as a single convex lens with an equivalent focus. The ratio between the size of the image formed by a lens and the size of the object gives the magnification of the lens. This is practically found by the ratio between the distance of the image from the centre of the lens

and the distance of the object from the same point. This latter distance in the case of the objective is approximately its focal length and the former the optical tube length. In practice, however, the mechanical tube length is taken instead of the optical tube length for purposes of calculating magnification. Hence, the ratio is finally reduced to that between the mechanical tube length and the focal length of the objective. For example, the magnification of the 2 mm. (1/12 inch) oil immersion objective corrected to a tube length of 160 mm., *i.e.* the initial magnification, is 160 divided by 2 which is 80. Using a 10× eyepiece, the total magnification is 80×10 or 800. Strictly speaking, the 2 mm. oil immersion lens has a shorter focal length and so it is more powerful, giving a magnification of 100 so that with a 10× ocular a thousandfold total magnification is obtained.

From the foregoing considerations it will be evident that a change in magnification can be brought about not only by changing the objective or changing the eyepiece but also by altering the distance between the objective and the eyepiece. In other words, the total magnification depends upon three factors: the object glass, the eyepiece and the tube length. The best method of increasing magnification is by using an objective with a higher power and higher aperture. Increasing the power of the ocular or enhancing the tube length by extending the draw tube does not affect the light gathering capacity of the objective.

Numerical Aperture. The perception of fine details of an object viewed depends upon the amount of light entering the objective from every point on the object; the wider the cone of light the better the result. That is, the aperture or the angular magnitude of the cone of light entering the objective from any point on the object should be wide. It is evident that the diameter of the **collecting** lens also serves as a measure of this cone of light, directly indicating the light gathering capacity of the objective. Usually the aperture is expressed by the product of the sine of half the aperture angle and the refractive index of the medium occupying the space between the object and the objective, *i.e.* $n \times \sin u$, where n stands for the refractive index and u for half the aperture angle. Abbe called the figure thus obtained the *numerical aperture* or N.A. The N.A. may be expressed as $n \times \sin u = n \times \frac{\text{half the diameter of the lens}}{\text{hypotenuse}}$.

With objectives of very short focal lengths, the focal length and the hypotenuse may be taken as almost equal and so the ratio becomes N.A. $= n \times \frac{\frac{1}{2}\text{ dia.}}{F}$. The maximum possible size for the aperture angle is reached when the front lens is almost touching the object. The value of it then is very nearly two right angles and u is 90°. As u can never be more than 90° and sin u is unity, the maximum N.A. for dry lens is one. In practice, however, this theoretical maximum can never be achieved and the highest obtainable for dry lens is 0·95. It is at once obvious from the above equation that the only possible way to obtain from a lens a numerical aperture greater than unity is to enhance the value of n by placing between the lens and the object a medium which has a refractive index higher than that of air. Because of its close resemblance to glass in its optical qualities, cedar wood oil is the medium usually used; hence the name oil immersion lenses for those that are designed to work in this way. As cedar wood oil has a refractive index of 1·5, approximately the same as that for glass, by the interposition of this the theoretical maximum for immersion objectives is 1·5, but the practical obtainable is only 1·40.

The numerical aperture provides a reliable criterion of all the essential qualities of an objective. For a given magnification, other things being equal, the intensity or brightness of the image varies as the square of the N.A., the resolving power and the defining power are directly proportional to the N.A., while the depth of focus, or the power of penetration, varies inversely proportional to it. Two objectives may have the same power of magnification but different numerical apertures, depending upon the diameter of the lens, when the one with the higher N.A. is the better objective as it admits a wider cone of light and so possesses greater resolving power. Cedar wood oil also improves the resolving power as it prevents deviation and consequent wastage of the peripheral portion of the rays of light proceeding up from the object. The figures representing the N.A. are inscribed on the objectives.

Resolving Power. The resolving power of a microscope is its capacity to distinguish clearly between two adjacent points. It is determined by the shortest distance between these points. It is this property that enables us to observe fine structural

details. Resolution is different from visibility; the latter does not include distinctness. The factors on which the resolving power of a lens depends are its N.A. and the texture of the light employed. The resolving power, as mentioned above, is directly proportional to the N.A. The resolving power of a microscope is limited by the wave length of the light used. The finer the wave length the better the resolving power and the finer the particles rendered visible. The limit of resolution is reached when two points on the object are separated by less than half the wave length of the light rays used. It can be expressed as $\frac{\frac{1}{2}\lambda}{\text{N.A.}}$. The human eye is most sensitive to green light which has an approximate wave length of 5,500 Å.U. When daylight is the source and the objective used is one with the highest N.A., 1·40, the theoretically possible limit is $\frac{\frac{1}{2} \times 5{,}500}{1 \cdot 4}$ or approximately 2,000 Å.U. or 0·2 micron. That is, the greater the N.A. the smaller is this distance and the smaller this distance the finer the details that can be resolved, or, in other words, the better the resolution. It goes with it that the higher the N.A. of an objective the finer are the particles rendered visible by it and with greater clearness of structural details. It should be noted that beyond the limit of resolution further magnification can still be obtained by using higher oculars, rendering perception possible, but the image so obtained is not clear and the additional magnification is, therefore, useless. In practice, with ordinary light, the limit of resolution would be reached at 0·25 micron. At this limit the magnification would be in the neighbourhood of 1,450 diameters. The effective magnification obtainable by a microscope lies between 500—1,000 times the value of the N.A. of the objective. The best dry lens can thus give an effective magnification of 950 (0·95 × 1,000), while the immersion lens with N.A. 1·40 can give 1,400. The superiority of the immersion lens is thus quite obvious.

Definition is the property of the objective which enables the formation of an image with a clear and distinct outline. What may cause the contrary is the spherical and the chromatic aberrations. The degree of definition, therefore, depends upon the degree of correction of the objective in respect of these two errors. A hundred per cent. colour correction is possible

only by the use of monochromatic light, but very great approximation to it has been achieved in the construction of the apochromatic objectives, referred to in greater detail in a previous section.

Fluorescent Microscopy. Fluorescent materials possess the ability to transform the invisible ultraviolet radiations into luminous rays and appear as self-luminous objects in their presence. Fluorescent microscopy takes advantage of this phenomenon. Transparent materials which are not naturally fluorescent are rendered fluorescent by a process of selective staining with some fluorescent solution. Auramine, berberine sulphate and fuchsine are some of the substances suited for this purpose. In this stained state irradiation with ultraviolet rays brings them out as brilliantly luminous objects in a dark field. This method has been applied with considerable success in the detection of acid-fast bacteria in specimens. But special equipments are necessary due to the filtration of ultraviolet light by glass and so the method has not come into general use.

Dark Ground Illumination. Nearly every organic structure is transparent to visual rays. Some are partially transparent while others are entirely so. Though transparent, they also reflect light under certain conditions. In the case of certain types of organisms, *e.g.* the spirochætes, the degree of transparency is too great to permit clear and direct observation by transmitted light. A special method of illumination whereby we are enabled to see such bodies is called the *dark ground illumination.* The underlying principle is Tyndall effect, well illustrated by the visibility of minute particles of dust in a shaft of light projected across a dark room.

In this method a powerful source of light, such as the arc lamp provided with a clock-work feed and a condensing lens or the pointolite lamp, arranged at a suitable distance, illumines the object by means of the plane mirror and a special type of condenser called the *dark field condenser.* The virtue of this condenser is that no direct light is allowed to enter the objective. The central portion of the upper surface of the condenser is made opaque to light, thus permitting only the oblique rays to pass up through the peripheral circular portion. The hollow cone of light that is so arranged is focused on the object. These incident rays are scattered from the surface of the object

under observation and the various points scattering light appear as brilliant sparks. Thus, in this method objects are rendered luminous by reflected and not by transmitted light. The central opacity is brought about by blackening; consequently objects appear as brilliantly lighted up images on a dark background. Hence the name dark ground or dark field, as opposed to bright field, for this method of illumination.

The special condenser may be of the paraboloid or of the concentric spherical reflecting type. What is essential for best results is that it should possess the same optical qualities as the objective. It is also essential that the condenser is accurately centered by means of the centering screws. The ordinary 2 mm. immersion lens is not suitable for this kind of work, as the amount of light it admits is too much. Its N.A. has to be reduced to less than 1·0 either by means of a vulcanite stop or a diaphragm interposed between the component lenses of the objective. The 1/7 inch fluorite immersion lens of N.A. 0·95 is very useful for routine D.G. work.

The glass slides used to mount the specimens must be of certain definite thickness, 1·0—1·1 millimetre. If not, the object would be either beyond or within the focus of the condenser. No. 1 cover-slips only should be used for this purpose. Care should be taken to make the preparation as thin as possible so that the object under view shall, as far as possible, be in one plane. Otherwise, the contrast will suffer. Too many objects in the field would also act as a defect by impairing contrast owing to a large number of objects scattering light. It is advantageous to seal the preparation with vaseline and prevent evaporation. A drop of oil free of air bubbles is placed on the surface of the condenser which is then slightly racked down. The preparation is mounted on the stage and the condenser raised home to effect the greatest proximity with the under surface of the slide. The beam of light from the microscope lamp, which is placed about 8–10 inches in front of the microscope, is now directed towards the centre of the mirror and reflected upwards towards the condenser. The preparation is then looked through the 16 mm. objective. On careful focusing, the centering ring, which is engraved on the top lens, will appear as a birlliant patch of light in the field. The condenser is correctly centered when this patch of light is in the exact centre and with the

maximum brilliance. It is a delicate piece of work to centre the condenser and, where circumstances permit, a microscope suitably arranged must be set apart permanently for dark field work. Having obtained the correct position, a drop of oil free of air bubbles is deposited on the cover-slip and the oil immersion lens turned on and carefully focused. Illumination is effected by oblique rays and objects are rendered visible not by transmitted but by reflected light. The course of the illuminating rays after they enter the condenser is through a homogeneous system. The suspended objects in the preparation appear as scintillating bodies on a dark background.

After the examination is over, the preparation should be dropped into a receptacle containing some antiseptic, set apart for the purpose. The oil from the optical parts should be carefully removed before leaving the microscope.

Micrometry. The unit of measurement in microbiology is the micron or μ which represents a millionth, or 1×10^{-6}, of a metre. A micro-micron or $\mu\mu$ is one-millionth of a micron or 1×10^{-12} metre. One Ångstrom unit (Å.U.) is 1×10^{-10} metre, and 10 Å.U. make one millimicron or $m\mu$, *i.e.* 1×10^{-9} metre. One thousand $m\mu$ make one μ and a thousand microns make one millimetre. Of these, the bacteriologist is concerned mostly with the micron and the millimicron. A conception of the size of a micron can be obtained from the knowledge that ordinary smoke is constituted of particles measuring about $0\cdot3\,\mu$ and the fine tobacco smoke of particles measuring about $0\cdot1\,\mu$ and this is about 1/2,000 of the diameter of human hair.

In practice, the size of microscopic particles is measured by the aid of a stage micrometer used in conjunction with a micrometer eyepiece. The stage micrometer consists of a slide 3×1 inch with a millimetre scale in the centre divided into hundred divisions so that one division is equal to $10\,\mu$. This is used to calibrate the actual value of the scale in the eyepiece. The micrometer eyepiece is a special type of eyepiece having a graduated scale mounted on a diaphragm. This is a purely arbitrary scale and has to be calibrated with reference to the micrometer scale to establish its real value. With any objective and the micrometer eyepiece working at a certain tube length the number of divisions on the eyepiece scale corresponding to one division of the stage scale can be easily determined. From this

the number of one division on the eyepiece scale in terms of microns can be calculated. Now substitute the object slide in place of the scale slide and, using the same objective and tube length, note the number of divisions on the eyepiece scale which covers the length or breadth of the object. The measurement of the object can then be deduced by multiplying the value of one division by the number of divisions observed to cover the size.

When viewed through a 2× micrometer eyepiece and a 2 mm. objective at a tube length of 160 mm., the 100 divisions of the eyepiece scale are found to tally with 17 divisions of the stage scale. Each division of the latter is 10 microns. Therefore, one division of the eyepiece scale is equal to 1·7 microns. If, for example, two divisions of the eyepiece scale cover the polar distance of a bacillus under investigation. the length of the bacillus is 2×1·7 or 3·4 microns. Only the micrometer stage scale has a definite value and it must be remembered that the value of the divisions of the eyepiece scale vary with the objective and the tube length employed. Therefore, whenever measurements have to be made with different objectives or with a different tube length, the value of a division of the eyepiece scale should be freshly determined for the particular combination.

Ultraviolet Microscope. Remarkable improvements have been made in microscopy in recent years. But there are limits to the possibilities of the light microscope. With ordinary light the compound microscope may in theory magnify an object to any extent, but in practice beyond a certain limit resolution suffers and any further increase in magnification becomes unprofitable. It has already been pointed out that this limit is reached when two points on an object or two objects in a field are separated by less than half the wave length of the light employed. In the case of visible light, the limit is reached theoretically when this distance is less than 0·2 and practically when less than 0·25 micron. That is to say, objects measuring less than 0·25 micron cannot be resolved even by the best compound microscope.

It has been explained before how the resolution of a microscope depends not only on its aperture but also on the wave length of the light used for illumination. The finer the wave length of light employed the greater the resolution and with it the greater the effective magnification possible. The ultraviolet rays have shorter wave length than the visible rays. Hence,

by using the former the resolving power of a microscope can be greatly enhanced, enabling finer particles, which are beyond the resolving power of the compound microscope, to be magnified and discriminated. But the problem is that the retina is insensitive to such small length radiations and the image formed is invisible. This difficulty has been overcome by substituting sensitive plates for the retina and taking photographic record of the image. Also, glass lenses are opaque to ultraviolet light. Hence, quartz lenses, which are transparent to this form of radiation, are used in place of glass lenses and a quartz prism acts as the mirror. The condenser, which should be also of quartz, is modelled as for dark field illumination. Special mounting slides and immersion fluids (mixture of water and glycerine or liquid petrolatum) are required. A mercury vapour lamp provided with quartz tube is a satisfactory source of ultraviolet light. The ultraviolet microscope has been extensively used in the past in the study of viruses.

Theoretically, it may be possible to extend the scope of a magnifying apparatus by utilising still finer rays than the ultraviolet radiation. But in practice, no substance is known to exist, which will transmit such rays. Lenses made of matter reach the limit of permeability with the ultraviolet microscope. But man's ingenuity has surmounted even this impasse by devising artificial lenses not containing matter.

Electron Microscope. In the electron microscope a stream of electrons is used in place of light. Electrons in motion behave as if they were associated with a wave length which is of the order of 1/100,000 of that of the visible light. This extreme shortness of electron waves endows the electron microscope with remarkably great resolving powers. When the stream of electrons traverses axially symmetrical electrostatic or magnetic fields, the latter act as matterless lenses (electrostatic or magnetic lenses), acting on the electron waves just as glass lenses will act on light waves. These lenses can be focused like the optical lenses, regulating resolution and magnification. But the focusing is done not by altering the relative distance of the objective and the specimen, as in light microscope with its fixed optical eyepieces, but by varying the lens power by increasing or decreasing the voltage of the current employed to produce the lens. The object is interposed in the path of the flowing

electrons which are focused by the object lens coil, causing primary magnification. A second magnetic lens can then be similarly arranged to function as an eyepiece and amplify the primary image. Thus, a very much magnified shadow image of the object is formed. But it is invisible and the eye is, therefore, unserviceable for its direct perception. Hence, it is observed on a fluorescent screen or photographed and studied later. The component parts of the electron microscope are all analogous to those of the optical microscope—source of light, condenser, objective and ocular.

The electron microscope has its limitations. Necessarily it is several times more expensive than the compound microscope and is available only for research and investigation. The object under observation is mounted as a dry film on an extremely thin, not more than a micron thick, collodion membrane supported on a fine mesh metal screen. In both ultra and electron microscopes no staining of the object to produce contrast is necessary, as almost every organic structure is opaque to the radiations employed. Since air will deflect the electronic waves which do not readily traverse matter, the instrument should be operated in a high vacuum chamber. In spite of these limitations and although electron microscopy is still in its infancy, it can be safely foreshadowed that its possibilities are immense. The magnification so far achieved is about 100,000 diameters, *i.e.* about 50 times the performance of the best light microscope.

In bacteriology the electron microscope is of value in the study of viruses, bacteriophages, the structure of bacteria, the mechanism of antigen-antibody combination and similar problems.

CHAPTER IV

MICROSCOPIC STUDY OF BACTERIA

The microscopic study of bacteria is carried out in the unstained living state as well as after staining with solutions of organic colouring substances called dyes. For such studies preparations of the materials containing bacteria are made on glass slides with or without employing cover-slips. The slides in common use measure 3 inches by 1 inch and should be of good white crown glass. The cover glasses may be round or square; rectangular ones are used for blood films. Those in general use are the "No. 1" cover-slips with a thickness ranging between 0·15 and 0·22 mm. The slides and cover glasses should be carefully cleaned and all grease got rid off before use.

Cleaning of Slides and Cover-slips. New slides are allowed to remain in alcohol containing 5 per cent. hydrochloric acid for a few hours. They are then washed in running water, spread on a clean towel, dried and stored in a stoppered jar containing 50 per cent. spirit. Used slides are thrown into a jar containing 3 per cent. lysol and allowed to remain there for about a week. After a preliminary washing in running water they are boiled for half an hour in soap suds. Thereafter they are thoroughly cleaned in cold water, dried and preserved as above. Before use slides are dried with a soft clean cloth. Cover-slips are dropped one by one into a glass beaker containing a 10 per cent. solution of chromic acid and boiled for about twenty minutes. They are transferred to a porcelain dish and washed thoroughly in running water until all traces of yellow colour have vanished. They are then washed in distilled water and rectified spirit in succession and finally transferred to a stoppered wide-mouthed bottle containing absolute alcohol wherein they are stored.

The usual mode of studying living micro-organisms is by what is called a *hanging-drop* preparation. The best results are obtained by this method, though often it may be enough to examine a drop on an ordinary slide under a cover glass. In the

hanging-drop method special slides with a central circular depression or concavity, called hollow slides, are employed. The depression is about one centimetre in diameter with a depth of about one-third the thickness of the slide. The material containing bacteria may be fluid, like urine or a fluid culture. If the organism is in solid culture, a suspension of it is made in physiological saline solution or broth, but fluid cultures are preferable to such emulsions. Examination for motility is best conducted with young cultures, not older than twenty-four hours. A small drop is placed on the centre of a clean cover glass and a hollow slide, with the edge of the hollow lined all around with vaseline, is applied to the cover glass with the hollow downwards. It is then suddenly inverted. The drop now actually hangs into the concavity of the slide from the under surface of the cover-slip; hence the name hanging-drop preparation. The vaseline serves the purpose both of sticking the cover-slip to the slide and preventing evaporation of the drop.

It is better to examine a hanging-drop preparation on a horizontal stage. The condenser is put out of action by lowering or removing. Light is focused properly with the concave mirror and the drop is examined with $\frac{2}{3}$ inch objective. The drop is moved about till the edge is seen as a silvery curve, which is then correctly centered and focused. Thus, a thin layer of the culture fluid is under view, which is preferable to examining the thicker central layers. Now the objective is changed to the $\frac{1}{6}$ inch and focused properly. It is not necessary to examine a hanging-drop by the oil immersion lens, as all the information that can be obtained by this method can be gathered by the high power dry objective.

The main object of this method is to study the motility of organisms as well as their natural arrangement. Care must be taken not to confuse Brownian movement for motility. The former is only an oscillatory movement within a very limited range shown by any suspended organic particle and caused probably by the impact of the surrounding molecules constituting the suspension fluid. Motility is a movement of translation and is evidence of life activity. Motile organisms move about from one part of the field to another. Non-motile organisms display Brownian movement to a greater or less extent and the beginner

is apt to mistake it for motility. The degree of motility and the type of motility are additional points that should be observed in the hanging-drop. The natural arrangement of organisms has also its diagnostic significance. Other features of morphology, such as shape, size, capsulation, spore formation and the like, are better studied after staining.

The hanging-block is a preparation devised for the microscopic study of the process of cell division. A small block of nutrient agar, about 8 mm. square, is cut out aseptically from an agar plate with a sterile scalpel and the centre of the surface inoculated lightly with a bacterial culture. It is dried for about ten minutes in the incubator. A clean sterile cover glass is applied on the inoculated surface and sealed by melting the agar round the edge with a hot needle. The preparation is sealed in the chamber of a sterile hanging-drop slide with paraffin and observations are made under the microscope.

The supravital method of staining, usually employed for the study of cells which are removed from the body and are still living, may likewise be used for the study of living bacteria. A drop of a stain, like methylane blue or neutral red, is spread on a clean slide and allowed to dry. A drop of the bacterial emulsion to be examined is then placed on the prepared slide and covered with a clean cover-slip which may be sealed with vaseline. The organisms are thus stained by the dye employed. The advantage of this method is that the organisms are stained without being subjected to the damaging action of fixatives.

Most of the bacteria can be studied in the living state by direct microscopy. This method, however, is not suitable for the examination of certain types of organisms. A special method designed for the examination of such organisms is by dark ground illumination.

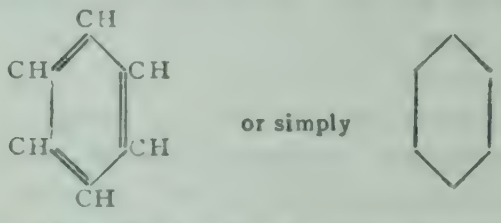
Finer organisms than bacteria, which are beyond the resolving power of the ordinary compound microscope, are studied by the ultraviolet and electron microscopes. But these are unsuited for routine use. Brief reference to these special methods is made in the chapter on microscopy.

Staining of Bacteria. The bacterial cell is almost colourless and nothing but the outline can be clearly seen when viewed under the microscope. In order to study the structural details it is necessary to stain bacteria. Staining is also useful in that

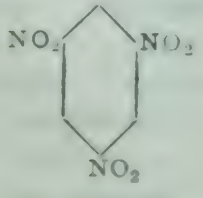
it provides a method of identification and classification of certain micro-organisms which possess special staining properties, *e.g.* the tubercle bacillus. It also enables the preservation of permanent specimens. The staining of bacteria is done with certain colouring organic compounds known as dyes or stains.

Bacterial Dyes. Originally the stains employed in bacterial staining were all derived from natural dyes. Artificial or synthetic dyes soon replaced them. They were manufactured from aniline and so they were called aniline dyes. Now practically all of them are manufactured from coal-tar derivatives and bear no direct relation to aniline. Hence, the term coal-tar dyes would be more accurate than the term aniline dyes.

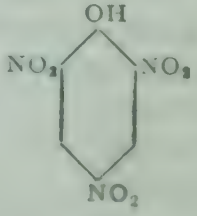
Most of the dyes are derivatives of the hydrocarbon benzol and contain one or more of these rings with chromophore and auxochrome groups linked to them. The hydrogen atoms of



benzol are replaceable and when one hydrogen atom is substituted by a chromophore group, like (NO_2), (NO) or (N = N), the compound becomes coloured. Such coloured compounds containing chromophore groups are called chromogens. As they do not possess the property of electrolytic dissociation, they cannot function as dyes, even though they are coloured substances. They are devoid of any affinity for bacteria, tissue cells or fibres and do not stain them. The colouring property can be imparted to a chromogen if another radical called the auxochrome group is introduced into the structure of the chromogen. What the auxochrome group does is to bring with it the salt forming property. For example, when three hydrogen atoms in



Trinitrobenzene



Picric acid

the benzene nucleus are replaced by three nitro groups (NO_2), the resulting compound is trinitrobenzene which is an yellow chromogen. This compound is not soluble in water and so, though yellow in colour, is not a dye. Now if an auxochrome group, such as (OH), is introduced into this compound in place of another replaceable hydrogen atom, the resulting compound is not only yellow in colour but also dissociable and therefore a dye. It is the common picric acid and its dyeing properties are due to the presence in it of the auxochrome radical (OH). It is, therefore, essential that a dye should have in its structure not only the chromophore but also an auxochrome group.

The auxochrome group may be acidic (OH) or basic (NH_2). Depending upon which group is present, the dyes are classified as acidic dyes or basic dyes. Commercial dyes are, strictly speaking, salts and not acids or bases. Acid dyes are sold in the form of salts of sodium, potassium, ammonium or calcium and basic dyes as salts of colourless acids, like hydrochloric, sulphuric and acetic acids. Basic stains display great affinity for the nuclear material. For this reason, they are generally employed in the staining of bacteria. The common stains used for bacteria are methylene blue, gentian violet, methyl violet and basic fuchsine. The acidic stains are chiefly used in the staining of cytoplasm: eosin, acid fuchsine, brilliant green and picric acid are a few examples.

Dyes are also used in bacteirology for other purposes than staining. Many of them possess antiseptic properties and are used as such. They are used in the preparation of selective media. The chromophore group in a dye can be easily reduced and in this process it loses its colour. In virtue of this property dyes are used as indicators. Some of the dyes are also valuable therapeutic agents.

Mechanism of Staining. The explanations given about the mechanism of staining are far from satisfactory. Two theories have been advanced, the physical and the chemical. According to the physical theory no new substances are formed in the process of staining of the bacterial protoplasm by the dye. This theory seeks to explain all the processes involved in staining on the basis of mere physical phenomena like osmosis, absorption and adsorption. A purely physical theory, however, fails to explain the mechanism of staining.

The protagonists of the chemical theory, on the other hand, contend that the entire mechanism of staining cannot be satisfactorily explained by mere physical processes. They assert that, as the result of the interaction between the bacterial protoplasm and the dye, new compounds are formed. No satisfactory proof has been brought forward in support of this theory. There is no evidence of the formation of any new substance during the process of staining and the dye can be recovered from the stained bacteria by the simple process of solution with suitable solvents. However, it is likely that both physical and chemical processes are involved in the process of staining.

Other agents employed during staining are intensifiers, mordants, fixatives and decolorisers. Certain substances like alkalies, acids and aniline oil, when added to the stain, enhance the latter's staining effect. Such agents are called intensifiers. The application of heat, in many cases, will improve the staining quality. In some cases bacterial cells are very difficult to stain; they have little or no affinity for the dyes employed. The addition of certain substances to the stain, however, promotes staining in such cases. Such substances are called mordants, for example phenol. Mordants possess strong affinities both for the substrate as well as the dye and in virtue of it fix the dye to the bacterial body. Fixatives are substances employed to fix the film to the slide or cover-slip and to harden tissues. The fixatives commonly employed are methyl alcohol, absolute alcohol, formalin, saturated aqueous solution of mercury perchloride and Zenker's fluid. In the case of films heat will serve this purpose quite well. Decolorising agents generally used are mineral acids, alcohol and acetone.

The proper making of films is very important for obtaining the best results. With a platinum loop a very small portion of the material to be studied is spread usually on a slide; films may be made also on cover-slips. A large amount of the material spread all over the slide is quite unnecessary and inelegant. Care should be taken to make the film thin and even and this depends in no small measure upon the cleanliness of the slides. These should be cleaned completely free from all grease (p. 59). Otherwise, fluid spread on them will roll in small droplets without spreading. The preparation is then dried in air and fixed, usually by heat. The slide is held with the thumb and index finger with the film side up and passed slowly three times

over a Bunsen flame. Overexposure to heat should be avoided. The exposure is just enough for proper fixation when the slide is not too hot to be borne when applied to the back of the hand. Fixation causes coagulation of the albuminous material, which ensures the firm adherence of the film to the slide. The preparation is now ready for staining.

Fluid materials, like broth culture, pus, exudates, urine, sputum and the like, are directly spread with a platinum loop. With solid materials, like growth on solid media and motion, a drop of sterile normal saline or water is placed on the slide. After sterilising and cooling the loop, a minute amount of the material is removed to the drop, thoroughly emulsified and spread. Gentle touch on the surface of the growth will usually suffice to take a sufficiency of the material.

Simple Stains. Bacterial reaction to stain is very much the same as the reaction of nuclear material. Any basic dye in dilute watery solution can be used as a staining reagent. Those in common use are methylene blue, basic fuchsine and thionin.

Loeffler's Methylene Blue

Aqueous solution of potassium hydroxide, one per cent.	1 c.c.
Water	99 c.c.
Methylene blue, saturated alcoholic solution	30 c.c.

Films are stained for one to two minutes and sections for five minutes or longer. Methylene blue is a safe stain and does not overstain even when the time limit usually laid down is transgressed.

Dilute Carbol Fuchsine

Ziehl-Neelsen's carbol fuchsine (p. 71) ..	1 c.c.
Distilled water	19 c.c.

Films are stained for 15–20 seconds. Unlike methylene blue, carbol fuchsine is an intense stain and readily tends to overstain and blur details.

Carbol Thionin. This is an excellent stain for demonstrating in tissues organisms like plague and glanders bacilli.

Stock solution:

Thionin	1 gram
Phenol, 1 : 40 watery solution	100 c.c.

For use:

Stock solution	1 part
Distilled water	3 parts

(Filter before use)

Stain sections for 5–10 minutes.

Wash well with water.

Differentiate in water to which a few drops of acetic acid have been added.

Wash well with water.

Blot, dehydrate with absolute alcohol, clear in xylol and mount in Canada balsam.

Negative Staining. In this method it is not the bacteria that are stained but the background. A homogeneous coloured background is produced by the stain employed and on this the unstained organisms are seen as clear transparent objects: in Burri's Indian ink method, which was originally devised for the staining of *T. pallidum*, a small drop of Indian ink is placed on a clean slide and a drop of serum from a suspected syphilitic sore is also transferred to it. These are mixed and spread out, with a slide, as a thin uniform film and allowed to dry. On examination the spirochætes are seen as unstained, transparent objects on a dark background. Nigrosin is another substance used in the relief staining of bacteria. A similar effect is produced after staining capsulated and sporing bacteria by the ordinary methods; the capsule or spore is seen as unstained, transparent structures in contrast to the stained bacterial body and the surroundings.

Selective or Differential Staining. By simple staining all bacteria are stained alike and no differentiation is possible between organisms possessing the same broad morphology. But they have been found to exhibit differences in their staining reaction to some of the dyes. Taking advantage of this, certain important staining methods have been evolved empirically, which are of considerable assistance in the diagnostic identification and classification of organisms. Two such methods are the Gram's and the acid-fast.

Gram's Stain. This is a very useful and important stain originally devised by Gram. It is also the most widely used of all the differential stains. On the basis of their reaction towards this staining method, bacteria are classified into two groups: the Gram-positive and the Gram-negative (p. 69).

The principle of this method depends upon the fact that when stained with an aqueous solution of any of the basic pararosaniline dyes, such as methyl violet or crystal violet, and then treated with a solution of iodine in potassium iodide, some bacteria retain the dye on subsequent treatment with a decolorising agent, such as alcohol, aniline or acetone, the iodine solution exerting a mordanting action and fixing the dye, whereas others lose it. Those that retain the stain are spoken as Gram-positive and those that are decolorised as Gram-negative. In a mixture of both varieties the Gram-positive bacteria appear violet stained, whereas the Gram-negative ones cannot be seen. In order to render these evident, another stain, usually red, is applied. A stain employed like this is called a *counter stain* or *contrast stain.* The organisms rendered inapparent as the result of treatment with the decolorant take the counter stain, while the already violet stained ones are not affected by this procedure. Therefore, the final picture presented is that of the violet stained Gram-positive organisms appearing side by side with the pink stained Gram-negative ones, thus producing a differential staining effect.

The Gram-positive property is confined practically to certain bacteria and yeasts; no other living cell shows it. Moulds respond somewhat irregularly towards this reaction. Even under the same conditions, all the Gram-positive organisms do not display the same capacity to retain the primary stain and so are not stained uniformly well. Some are well stained and some are feebly stained. Certain conditions, such as age of the culture, action of tissue ferments, and the pH of the medium, influence the Gram reaction. For example, old cultures, organisms in pus, etc., stain irregularly. But such irregularities do not detract from the general differential value of the method. It is worth while remembering that a Gram-positive organism may lose the primary stain and appear as Gram-negative, whereas a Gram-negative organism will never appear as Gram-positive.

The mechanism of the reaction is by no means clear. Several theories have been advanced to explain it. The Gram-positive property is said to be a function of the intact cell membrane which does not permit the escape of the alcohol-soluble iodine-dye complex formed in the cell, whereas in the case of the Gram-negative organisms the cell membrane readily allows this. Other theories propounded to explain the differential reaction of

the two classes of bacteria are: difference in the state of intracellular dispersal of the bacterial protoplasm, difference in the intracellular pH and peculiarities on their chemical structure. Recently evidence has been brought forward in support of the last theory. A chemical substance, magnesium ribonucleate, has been extracted from the Gram-positive bacteria. Removal of this substance renders them Gram-negative, while recombination restores the Gram-positive property. Ribonucleate obtained from any source, such as yeast, will restore the lost property. But it is highly specific and even closely related compounds cannot replace it. No such substance can be obtained from the Gram-negative bacteria nor do these evince any tendency to combine with this chemical. Magnesium ribonucleate seems to reside in the cytoplasmic coating in combination with certain protein constituents. This combination appears to be essential for the Gram-positive quality.

The pararosaniline dyes are found most suitable for the primary stain in the Gram's method. They are methyl violet, crystal violet, gentian violet (which is a mixture of the other violet pararosanilines) and victoria blue; of these, methyl violet and gentian violet are the most commonly used ones in the Gram's technique. Several modifications have been made in the original method, but the principle involved is the same.

Jensen's Modification of Gram's Stain

Reagents

Methyl Violet:

Methyl violet (6B)	0·5 gram
Distilled water	100 c.c.

(Filter)

Iodine Solution (Lugol's iodine):

Iodine	1 gram
Potassium iodide	2 grams
Distilled water	100 c.c.

This iodine solution is three times stronger than the original Gram's iodine.

Neutral Red Solution:

Neutral red	1 gram
Acetic acid, 1 per cent.	2 c.c.
Distilled water	1,000 c.c.

Prepare the film and fix it as mentioned above.

Pour the methyl violet solution on the film and allow it to act for half a minute.

Hold the slide in a slanting position and wash off the methyl violet with the iodine solution. Allow the iodine solution to act for half to one minute.

Wash off the iodine with absolute alcohol, a few drops at a time, until no more stain comes off the preparation. This can be easily made out by noticing the colour of the draining fluid at the tip of the slide, which should have no more than a faint trace of colour.

Wash off the iodine solution with the counter stain and allow this to act for half to one minute.

Wash with distilled water and dry between blotting paper.

A 0·5 per cent. safranin or dilute (1 : 20) carbol fuchsine may be used in place of neutral red.

If properly done, all Gram-positive organisms and fibrin appear violet and Gram-negative organisms, tissue cells, pus cells and their nuclei appear pink with the counter stain.

TABLE I

Reactions of Certain Bacteria to Gram's Stain

Positive	Negative
Staphylococci ..	.. *Gonococcus*
Streptococci ..	.. *Meningococcus*
Pneumococcus ..	.. *N. catarrhalis*
Micrococci ..	.. *Br. melitensis*
Sarcinæ ..	.. *Br. abortus*
C. diphtheriæ ..	.. *H. influenzæ*
C. hofmannii ..	.. *H. pertussis*
C. xerose ..	.. *H. duplex*
Myco. tuberculosis	.. *Proteus*
Myco. lepræ ..	.. *Ps. pyocyanea*
Myco. smegmatis	.. *Past. pestis*
Actinomyces ..	.. *Pf. mallei*
B. subtilis ..	.. *V. choleræ*
B. anthracis ..	.. Coli-aerogenes group
Cl. tetani ..	.. Friedlander group
Cl. welchii ..	.. Enteric group
Cl. novyi ..	.. Salmonella group
Cl. septicum ..	.. Dysentery group
Cl. sporogenes ..	.. Vincent's bacillus
Cl. botulinum ..	.. **Spirochaetes**

The Acid-Fast Stain. Most of the organisms are readily stained by aqueous solutions of coal-tar dyes. There are, however, a few which are difficult to stain in this way. These organisms can be forced to take the ordinary stain by special methods and when once stained, they resist decolorisation with dilute mineral acids. The dye sticks fast to the organism even in the presence of the strong decolorising agent, the acid. Such organisms are, therefore, termed *acid-fast.*

The difficulty in staining was first encountered in the case of the tubercle bacillus and it was in connection with it that the acid-fast method was developed. Ehrlich found that when tubercle bacilli were stained with warm aniline oil basic fuchsine or aniline oil methyl violet, they resisted subsequent decolorisation with dilute mineral acids. Ziehl introduced phenol as a mordant and showed that a solution of basic fuchsine in 2 per cent. aqueous phenol gave better results. Neelsen subsequently increased the strength of phenol to 5 per cent. Hence, this method is known as the Ziehl-Neelsen method.

The acid-fast property is said to be due to the presence of a high percentage of lipoid material in the envelope of the acid-fast organisms, which probably prevents the penetration of the dye. An unsaponifiable waxy substance possessing acid-fast property has been isolated from them. All acid-fast organisms contain this waxy substance to a greater or less extent and the degree of acid-fastness depends upon the amount present. The greatest waxy content is found in the tubercle bacillus and it is strongly acid-fast; the saprophytic acid-fast organisms contain only very small proportions and they are feebly acid-fast. The property of acid-fastness is also a function of time. After the primary stain, if the organisms are exposed to the action of the acid for considerably long periods, they lose the stain. The acid-fast bacilli are also alcohol-fast in varying degrees, the pathogenic ones considerably more than the saprophytes.

Basic fuchsine is a powerful nuclear dye. With the mordanting action of phenol and the application of heat, the dye is able to penetrate into the cell. The heat employed probably melts the waxy material, facilitating more rapid and intimate staining. Prolonged staining without the application of heat also brings about the same effect. Just as the organism resists ingress of the dye, it also resists the dissolving out of the dye by the acid.

All other organisms are completely decolorised. In order to render such decolorised organisms visible, a counter stain, usually methylene blue, is used.

Ziehl-Neelsen Method

Carbol fuchsine:

Basic fuchsine	1 gram
Absolute alcohol	10 c.c.
Carbolic acid, aqueous 5 per cent. solution	100 c.c.

The dye is dissolved in alcohol first and then added to the phenol solution.

Staining:

Prepare the film and fix it over a flame as mentioned above.

Pour carbol fuchsine over the film and heat gently to steaming.

Allow the stain to act for five minutes, heating the slide at intervals. Complete evaporation of the stain and drying should be avoided; so also boiling.

Wash well with water, removing all the excess stain from the slide.

Decolorise with 20–25 per cent. sulphuric acid (5–10 per cent. for *Myco. leprœ*), washing alternately with water to remove all the acid-dye compound and allow fresh acid to act. Carry it on until no more colour is dissolved out and the smear after washing with water gives a faint pink tint.

Wash the slide thoroughly with water.

Counter-stain with Loeffler's methylene blue for one minute.

Wash and dry between blotting paper.

The acid-fast organisms appear bright red on a blue background, while tissue cells and other organisms are stained blue in contrast to the red acid-fast bacilli.

As contrast stains malachite green in one per cent. solution or saturated solution of picric acid may be used applying for 15–20 seconds. The former gives a pale green and the latter a yellow background.

Any mineral acid may be used for decolorisation, 20 per cent. nitric acid or 3–4 per cent. hydrochloric acid.

Some workers advocate treating the film with absolute alcohol for two minutes as a routine procedure after decolorisation

with acid. This is to decolorise weakly alochol-fast saprophytic acid-fast organisms like the smegma bacillus.

Staining of Section. All manipulations should be gentle; otherwise, the section is apt to be dislodged from the slide.

Remove paraffin by treating with xylol, wash with alcohol and then with water, as described on page 82.

Stain with carbol fuchsine as described above, but heat gently lest the section should get detached from the slide.

Wash thoroughly but gently with water.

Decolorise with 20–25 per cent. sulphuric acid (5–10 per cent. for *Myco. leprœ*). The process takes a longer time due to thickness of the section.

Wash well in water.

Counter-stain with methylene blue or malachite green for 30–60 seconds.

Wash well with water.

Wipe the slide dry all round the section. Pour a few drops of absolute alcohol, pour off the excess and press the section with blotting paper using steady pressure.

Immediately pour more absolute alcohol and complete the dehydration.

Clear in xylol.

Mount in Canada balsam.

Special Stains. Special stains have been devised for the demonstration of certain bacterial structures, *viz.* metachromatic granules, capsules, flagella and spores, which are either difficult to stain by the ordinary methods or give characteristic appearances by these special staining processes.

Staining of the Diphtheria Bacillus. The presence of metachromatic granules help in the identification of this organism. These granules are stained by any basic dye, but characteristic reactions are given by certain special staining methods. Young cultures, 18–24 hours, are to be used; otherwise, the presence of bizarre forms common to allied organisms will vitiate the results. Again, the characteristic appearance is given best when the growth is from media containing serum, such as the Loeffler's medium.

Neisser's Method (modified)

Solution A:

Methylene blue	1 gram
Absolute alcohol	50 c.c.
Glacial acetic acid	50 c.c.
Distilled water	1,000 c.c.

Solution B:

Crystal violet	1 gram
Absolute alcohol	10 c.c.
Distilled water	300 c.c.

Counter stain:

Chrysoidin	1 gram
Distilled water	300 c.c.

Dissolve by gentle heat and filter

or

Bismark brown	1 gram
Distilled water	500 c.c.

Mix together two parts of solution A and one part of solution B.

Flood the film with the mixture and stain for 30–60 seconds.

Counter-stain with chrysoidin or Bismark brown for 30 seconds.

Wash in water and dry.

The granules of the bacilli appear blue black, while the protoplasm stains yellowish brown.

Pugh's Stain

Toludine blue	2 grams
Absolute alcohol	20 c.c.
Glacial acetic acid	50 c.c.
Distilled water	950 c.c.

Prepare and fix the film as usual.

Cover the film with the stain and gently steam it for one to three minutes.

Wash in water and dry.

The granules are stained reddish purple, while the bacillary body takes a light blue tint. This is a very useful and rapid single stain.

Staining of Capsule

Hiss's Method

Reagents:

(1) Saturated alcoholic solution of gentian violet (or basic fuchsine) .. 1 part
Distilled water .. .. .. 19 parts
(2) Copper sulphate solution, 20 per cent. aqueous

Films should be thin and fixed by heat in the usual manner.

Cover the film with the stain and gently heat until steam arises.

Stain for 20–30 seconds.

Wash off the stain with the copper sulphate solution repeatedly with liberal amounts of it till the slide is quite cool. This prevents the excess deposit of copper sulphate crystals on the film.

Dry between blotting paper without washing and mount, if necessary, in Canada balsam.

The capsules are stained pale bluish purple and bacteria deep violet.

Muir's Method

Reagents:

Carbol fuchsine
Special mordant
Loeffler's methylene blue

Mordant:

Mercuric chloride, saturated aqueous solution .. .. 2 parts
Tannic acid, 20 per cent. aqueous solution 2 parts
Potash alum, saturated aqueous solution.. 5 parts

Very thin films are prepared and fixed by heat as usual.

Stain with carbol fuchsine for one minute, heating gently to steaming.

Wash slightly with spirit and then well with water.

Treat with mordant for 30 seconds.

Wash well with water.

Treat with spirit for 20–40 seconds till the film is pale red in appearance. The exact time depends upon the preparation and is to be determined by trial.

Wash well with water.

Counter-stain with methylene blue for 30–60 seconds.

Wash well with water and dry in the usual way or the film may be dehydrated with absolute alcohol, cleared in xylol and mounted in balsam.

The capsules are stained bright blue and bacteria deep crimson.

This method gives beautiful results but requires considerable experience and skill.

Staining of Flagella. Flagella are not visible when examined in a hanging-drop preparation; they may, however, be made out when examined by dark ground illumination. Ordinary methods of staining fail to show them up; special stains have to be employed for their demonstration. The staining of flagella is very difficult and demands a great deal of manipulative skill and care. Success also depends in no small measure on the cleanliness of the slides used and the way in which the films are made.

A small amount from an agar culture is emulsified in water, the quantity of culture being only just enough to cause the faintest turbidity of the water. This may also be done as follows: a drop of water is placed on a clean slide and a small speck of growth taken on the point of a platinum needle is held in contact with the surface of the drop of water and gently shaken for a few seconds, allowing the bacteria to swim off into the water. From the emulsion a thin film is made on a perfectly clean slide and allowed to dry in the air.

Muir's Modification of Pitfield's Method

Reagents

A. Mordant:

Tannic acid, 10 per cent. aqueous solution	10 c.c.
Corrosive sublimate, saturated aqueous solution	5 c.c.
Alum, saturated aqueous solution ..	5 c.c.
Carbol fuchsine	5 c.c.

Mix well. A precipitate forms. Centrifugalise or allow it to settle. Remove the clear supernatant fluid with a pipette and transfer it to a clean bottle. This will keep for one or two weeks.

B. Stain:

Alum, saturated aqueous solution ..	10 c.c.
Gentian violet, saturated alcoholic solution	2 c.c.

Freshly prepared stain should be used, at any rate not one more than two or three days old.

Technique:

Flood the prepared slide with the mordant. Heat gently until steam rises and allow steaming for one minute.
Wash well in a gentle stream of running water for two minutes.
Dry carefully over a flame.
Cover the film with the stain, steam gently for one minute.
Wash well in water.
Dry and mount in Canada balsam.

Staining of Spores. When spore-bearing organisms are stained by ordinary methods, the bacillary body is stained leaving the spore unstained as a clear area in the organisms. This is probably due to the resistant envelope of the spore preventing penetration by stains. Spores may, however, be stained by special methods. Once the spore is stained, it resists decolorisation with great tenacity, recalling the behaviour of the tubercle bacilli.

Moeller's Method

Prepare and fix films in the usual manner.
Treat with chloroform for two minutes.
Wash in water.
Treat with 5 per cent. chromic acid for 2 minutes.
Wash in water.
Pour carbol fuchsine and heat gently, steaming for 3–5 minutes.
Decolorise with 5 per cent. sulphuric acid or methylated spirit for 5–10 seconds.
Wash in water.
Stain with 1 per cent. aqueous methylene blue for ½–1 minute.
Wash in water, dry and mount in balsam.

The spores are stained bright red and the bacterial body blue.

The Romanowsky Stains (Polychrome Stains). The interaction of methylene blue and water-soluble eosin leads to the formation of a compound which imparts a reddish purple colour to the chromatin of microbes. This is the basis of preparing all Romanowsky stains, such as Leishman's, Wright's, Jenner's, and Giemsa's. Methylene blue is "ripened" before mixing, either by age or by heating with sodium carbonate. On mixing the ripened methylene blue and eosin, a precipitate is formed, which is washed with distilled water, dried and preserved. For use the dry product is dissolved in acetone-free methyl alcohol. The difference between the various modifications of the Romanowsky stain depends upon the relative proportions of methylene blue and eosin, as also on the method of ripening of methylene blue. The method of ripening employed in the preparation of Leishman's and Wright's stains, for instance, is by heating with sodium carbonate.

All these stains are obtainable both in powder form and in solution ready for use. They deteriorate rapidly on exposure to sunlight and so should be preserved in the dark. Unless the prepared stains are kept in glass stoppered bottles, they may become concentrated by the slow evaporation of the alcohol.

Leishman's stain is prepared by dissolving the dry powder in acetone-free methyl alcohol in 0·3 per cent. strength. Some of the blood spirochætes are readily stained by this stain and the technique of staining is the same as for staining the malarial parasite. The film need not be fixed as the methyl alcohol acts as a fixative.

Giemsa's stain consists of a number of compounds designated Azure I, Azure II and Azure II-eosin. These are formed by the interaction of varying proportions of methylene blue and eosin. The ready made commercial stain is better than that made in the laboratory. Giemsa's stain is used in two ways: the rapid method and the slow method. In both cases staining is preceded by fixation of the film either by methyl alcohol for three minutes or absolute alcohol for fifteen minutes.

Rapid Method. This may be used in all cases where Leishman's stain is used.

Fix the film in methyl alcohol for three minutes.

Cover it with the stain and add double the volume of distilled water, mix and allow to act for five minutes.

Wash in distilled water.

Dry between blotting paper.

Slow Method. This is employed in the staining of objects, like the spirochætes, which are difficult to stain by the ordinary methods. The film is fixed with methyl alcohol for three minutes. It is laid in a Petri dish with one end raised on a piece of glass rod and with the film side down, so as to prevent any deposit of stain on the film. Dilute stain (1 : 10 of distilled water) is then poured into the Petri dish. The dish is covered and the stain allowed to act for 16–24 hours. The slide is washed and dried as usual.

Staining of Spirochætes

Fontana's Method

Reagents

Fixative:

Glacial acetic acid	1 c.c.
Formalin	20 c.c.
Distilled water	100 c.c.

Mordant:

Carbolic acid	1 gram
Tannic acid	5 grams
Distilled water	100 c.c.

Ammoniacal silver nitrate: Add 10 per cent. ammonia to a 0·25 per cent. aqueous solution of silver nitrate until the precipitate formed just dissolves. Then add more silver nitrate solution drop by drop until the precipitate reappears and does not again go into solution. The solution is now just opalescent.

Technique:

Treat the film with the fixative for a minute or two, changing the fixative once or twice.

Wash thoroughly in running water.

Cover the film with the mordant and heat to gentle steaming; allow it to act for 30 seconds.

Wash thoroughly with distilled water and dry.

Treat with the silver nitrate solution, steaming gently for 30 seconds, when the film becomes brown in colour.

Wash well in distilled water, dry and mount in Canada balsam.

The spirochætes are stained dark brown or black due to the precipitation on them of minute particles of silver which also make them appear thicker.

Levaditi's Method of Staining Spirochætes in Tissues

Fix very thin slices (1 mm. thick) of tissue in 10 per cent. formalin for twenty-four hours.

Wash in water for one hour.

Place in 96–98 per cent. alcohol for twenty-four hours. Remove them to a dark bottle containing 1·5 per cent. silver nitrate solution and keep in the incubator for three days.

Wash in distilled water for 30 minutes.

Place the pieces in the reducing solution made as follows:

Pyrogallic acid	4 grams
Formalin	5 c.c.
Water	100 c.c.

Allow them to remain in it for 48 hours at room temperature.

Wash well in distilled water.

Dehydrate with increasing strengths of alcohol and clear in xylol.

Embed in paraffin.

Cut thin sections, remove paraffin with xylol and mount in Canada balsam.

The spirochætes are stained black and the background pale yellow.

Staining of Inclusion Bodies. Giemsa's stain is satisfactory when these are basophilic in nature, *e.g.* in psittacosis. But, for acidophilic inclusion bodies more satisfactory stains have been devised.

Pieces of tissue are fixed in Bouin's or Zenker's fluid and paraffin sections cut in the usual manner.

Mann's Stain

Methylene blue, 1 per cent. aqueous solution 35 parts
Eosin, 1 per cent. aqueous solution .. 45 parts
Distilled water .. .. 100 c.c.
Stain for 12 hours in the incubator at 37° C.
Rinse in water.
Differentiate under the microscope in 70 per cent. alcohol with a drop of aqueous orange G. solution added to each cubic centimetre.
Dehydrate and mount in balsam.

Staining of Elementary Bodies. While elementary bodies may likewise be stained by the Giemsa's technique, more satisfactory results are obtained by other methods.

Paschen's Method

Loeffler's flagella mordant:

Tannic acid, 20 per cent. aqueous solution 100 c.c.
Ferrous sulphate, saturated solution .. 50 c.c.
Basic fuchsine, saturated alcoholic solution 10 c.c.
Make films from infected material on slides and let them dry.
Place in distilled water for five minutes and dry in air.
Fix in absolute alcohol for five minutes and dry in air.
Treat with the mordant, gently heating for one minute, and allow it to act for ten minutes.
Wash in distilled water.
Stain for ten minutes with 5 per cent. carbol fuchsine solution, gently heating during the first minute.
Rapidly wash in water, dry and mount in cedar-wood oil.

Castaneda's Method. This is a very satisfactory method for staining *Rickettsiæ*. It may be used for staining elementary bodies.

(*a*) *Buffer Solution:*

Potassium dihydrogen phosphate (KH_2PO_4) 1 gram in 100 c.c. of distilled water.
Sodium monohydrogen phosphate (Na_2HPO_4. 12 H_2O) 25 grams in 900 c.c. of distilled water.
(Mix and adjust pH to 7·5)
Add 1 c.c. of formalin as a preservative.

(*b*) Methyl alcohol .. .. .. 100 c.c.
Methylene blue .. .. .. 1 gram

To 20 c.c. of buffer solution (*a*), add 1 c.c. of formalin and 0·15 c.c. of (*b*).

(*c*) *Counter stain:*

Safranin "O", 0·2 per cent. in distilled water .. .. 1 part
Acetic acid, 0·1 per cent. .. .. 3 parts

The film is stained for three minutes. The stain is then poured off, without washing, and the smear is counter-stained with safranin for one to four seconds (never more). The film is then washed in running water and dried.

The rickettsiæ stain blue, while the cytoplasm and nuclei of the cells are stained red.

Staining of Bacteria in Tissues. Occasionally it may be necessary to examine tissues for the presence of bacteria. For this purpose, tissue is cut into thin sections and stained. It is quite essential for accurate results that the tissue examined should be as fresh as possible. Otherwise, the post-mortem changes that will ensue will interfere with the bacterial content and vitiate the results.

Thin pieces of the tissue, about $\frac{1}{8}$ inch thick, are cut and fixed or hardened in a suitable fixative. They are then dehydrated by several changes of alcohol, so that paraffin may permeate into the tissue. Dehydration is followed by treatment with a fluid, like xylol, benzol, acetone or chloroform, which is a solvent of both alcohol and paraffin. The tissue is then embedded in melted paraffin.

Fixatives. Formalin in 10 per cent. strength in physiological saline solution is a convenient and satisfactory fixative for general use. Tissue cut into thin slices is put into a large volume of fixative. The latter is changed once at the end of twenty-four hours; in another twenty-four hours fixation is usually complete. The pieces are then washed in running water for an hour and kept in 50 per cent. alcohol pending dehydration.

Zenker's Fluid:

Mercuric chloride .. .. .. 5·0 grams
Potassium bichromate .. .. .. 2·5 grams
Sodium sulphate .. .. .. 1·0 gram
Water .. .. 100 c.c.

Immediately before use, 5 c.c. of glacial acetic acid are added to every 100 c.c. of the fluid.

Thin pieces of tissue are placed in the fluid for 12–24 hours and then washed thoroughly in running water for 24 hours to remove the potassium bichromate and mercuric chloride. They are then transferred to 50 per cent. alcohol until used.

Bouin's Fluid:

Picric acid, saturated watery solution	.. 75 parts
Formalin	.. 25 parts
Glacial acetic acid	.. 5 parts

This is quite a satisfactory fixative for tissues meant for the study of inclusion bodies. It keeps well. Pieces of tissue are dropped in it for 1–12 hours according to their thickness and consistency. They are washed in 50 per cent. and 70 per cent. alcohol successively until the picric acid is removed.

Paraffin Embedding. The fixed pieces of tissues are put through as follows:

90 per cent. spirit for 2–5 hours.
Absolute alcohol for 2 hours.
Fresh absolute alcohol for 2 hours.
Mixture of equal parts of absolute alcohol and chloroform till tissue sinks or overnight.
Pure chloroform for 6 hours.
Mixture of equal parts of chloroform and paraffin, which is kept melted in the paraffin oven, for one hour.
Pure melted paraffin in the oven at 55° C. for two hours.

The tissue is then blocked and sections cut by microtome. The sections should not be more than 3–6 microns thick. After cutting they are spread out on warm water, floated on to slides and allowed to dry. Albuminised slides are preferable where staining involves heating.

The paraffin has to be removed from sections so that watery stains may penetrate into the tissue. The section is, therefore, passed through as follows:

Xylol for a few minutes (the slide may be gently warmed immediatey before this step).
Absolute alcohol, rectified spirit, 70 per cent. spirit in succession for a few minutes each.
Running water for some time.

Lugol's iodine, if the tissue is fixed in Zenker's fluid, for a few minutes.
Running water.
Rectified spirit.
Running water.

The section is now ready to be stained. After staining and washing well in water, the slide is cleaned all round the section with a piece of cloth or blotting paper. Add a few drops of spirit and drain. Place the slide on the desk and press the section under blotting paper using steady pressure. This removes the bulk of water in the section. Add immediately a few drops of spirit and complete the dehydration with absolute alcohol. Remove the alcohol with xylol and after clearing is complete, wipe off the excess of xylol round the section. Put a drop of Canada balsam and apply a cover-slip. Great care should be taken that the section is never allowed to dry at any stage of the process.

CHAPTER V

THE PREPARATION OF CULTURE MEDIA

Morphology alone does not form a trustworthy guide in the study and identification of micro-organisms. For such purposes bacteria are grown in artificial nutrient preparations called *Culture media.* The successful cultivation depends mainly upon the nutritive qualities of the culture media; the presence of moisture in adequate quantities is also an essential requisite. Bacteria vary widely in their nutritional requirements; it is not, therefore, surprising that a wide variety of media, no less than 7,000 different types, have been devised to meet the varied requirements. It is also necessary, if more than one type of organism are present, to isolate them in what are called *pure cultures*, so that their specific characters may be studied accurately. Many methods have, therefore, been devised for the isolation of individual species. It may be recalled that bacteria are ubiquitous and are usually present in all materials and articles used for the preparation of media. Hence, one of the essential prerequisites of all cultural methods is a preliminary removal or destruction of all forms of microbic life from the media employed for the growth of organisms. Further, the sterility thus created in culture media should be maintained by protecting them from extraneous contamination.

Generally, culture media are prepared and stored in glass containers of different shapes and sizes. Those in common use are test tubes, Petri dishes and flasks of both Erlenmeyer and the ordinary Florence types. These, of course, should be thoroughly cleaned and sterilised before use.

Recently screw-capped bottles have been introduced for the routine use and storage of culture media. They are available in varying shapes and sizes. They have the advantage that media stored in them undergo very little desiccation and keep sterile for a long time. They are also very convenient for long distance transport.

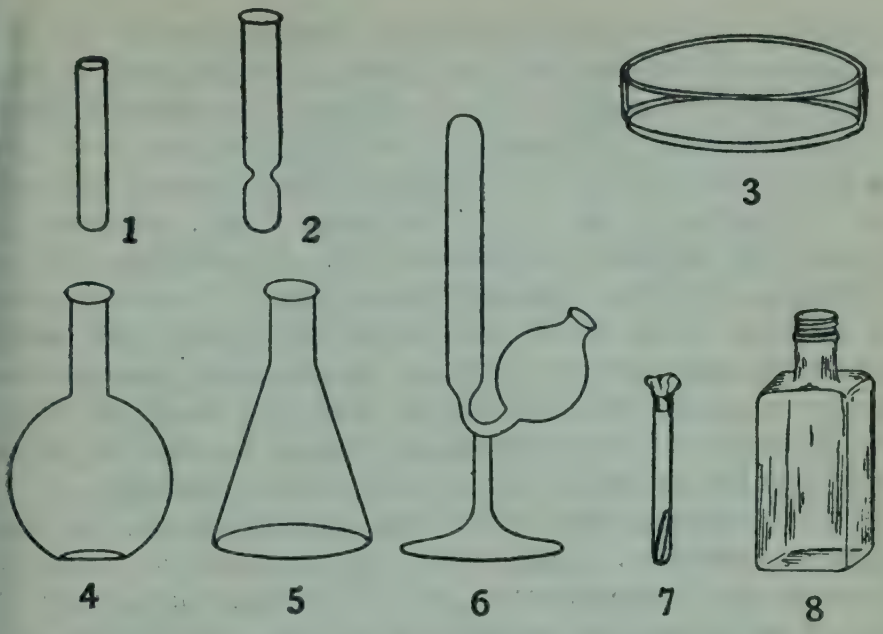


FIG. 4. 1, Test Tube; 2, Roux Tube; 3, Petri Dish; 4, Boiling Flask; 5, Erlenmeyer Flask; 6, Smith's Fermentation Tube; 7, Durham's Fermentation Tube; 8, Screw-capped Media Bottle.

Preparation of Glassware. Free alkali may be often present on new glassware. In order to remove it, they may be immersed in one per cent. hydrochloric or nitric acid for several hours. They are then treated with one per cent. sodium hydroxide for some time, which is followed by thorough washing in running water. Used glass containers with culture media are first sterilised either by immersion in three per cent. lysol for twenty-four hours or in the autoclave. The latter procedure is safer, especially when dealing with culture media containing such dangerous and hardy organisms like the tubercle bacillus or the spore-forming pathogens like the tetanus and anthrax bacilli. After sterilisation, they are boiled in five per cent. sodium hydroxide or in soap suds for fifteen to twenty minutes. This is followed by mechanical cleansing with suitable brushes and thorough washing in tap water. They are then allowed to drain and dry. The screw-capped bottles, as they are supplied after being subjected to special cleaning processes by the manufacturers, may be used without any elaborate treatment.

After drying, test tubes and flasks are closed with cotton wool plugs. This has been found to be quite efficient in preventing the entry of extraneous micro-organisms, commonly present in the air, by filtering them in their meshes. For this purpose,

long-fibre, non-absorbent cotton is generally used; the disadvantages of the absorbent type, especially when used in association with fluid media, are quite obvious. The plugging should be carefully done. Otherwise, it will defeat its own end. No creases or folds should be allowed to form between the plug and the surface of the glass; for these, if left, will serve as channels for the entry of unfiltered air. A convenient method of plugging is to break a piece of cotton, about 2–3 inches square, by catching at the centre with a pair of forceps and gently forcing the piece into the mouth of the glassware, leaving some portion to project out for handling. The plug should fit firmly, just allowing to lift the container by means of the plug, but at the same time not so tight as to render its removal difficult.

After plugging with cotton wool and before use, the containers are sterilised. This is best done in the hot air steriliser. Exposure to a temperature of 160° C. for one hour or 180° C. for half an hour ensures complete sterilisation. Shorter exposure to a higher temperature saves time; but care should be taken that the temperature is not raised above 200° C., for cotton plug begins to brown beyond this temperature and the burning of the grease and other materials derived from the cotton will cause staining of the glassware. Petri dishes, pipettes and the like are wrapped in kraft paper before sterilisation; as a measure against subsequent contamination, the paper is kept on until use.

Culture Media. The food requirements of micro-organisms have been dealt with in a previous chapter. These requirements are not the same for all bacteria and special media have to be provided for highly parasitic species. Nevertheless, there are certain basic requirements common for most species and on the basis of this certain standard media of general utility have been elaborated, for example nutrient broth (bouillon) and nutrient agar. In these, all but the most fastidious microbes grow, though with varying vigour.

Meat infusion constitutes the basis of many common media. It is a watery extract prepared by macerating lean beef or veal in distilled water and leaving the mixture in the ice chest overnight. Next morning it is strained through muslin. The infusion contains all the soluble constituents of meat, mineral salts, small amounts of sugar, some nitrogenous muscle extractives and colouring matter, but is devoid of any protein. Its

value as an ingredient of culture media is undoubted, but its exact properties have not been fully studied. Evidently it is not sufficiently nutritious by itself and so certain other substances have to be added to make up the necessary quality. Peptones, split products of protein digestion, are the most important of these. The soluble constituents of meat are also available in the form of certain commercial preparations called meat extracts, *e.g.* lemco. For general work, a 5–10 per cent. solution of lemco may take the place of meat infusion.

In the early days of bacteriology, fluid media were the only ones available, and these, of course, did not lend themselves for easy isolation of bacteria in pure culture. The development of experimental bacteriology was greatly hampered in those days by the severe limitations inherent in fluid culture. This, in its turn, acted as a great handicap in the scientific study of bacterial species. Koch (1881) was the first to introduce solid media for the growth and isolation of bacteria in pure culture. He did this by adding gelatin to the fluid media. Gelatin has, however, a serious limitation in that it melts at 24° C., making it unsuitable for the optimum growth of many delicate pathogens which thrive best at body temperature, 37·5° C. This difficulty found solution by the introduction of agar agar, a sea weed, which remains solid well above the temperature of the body. Gelatin is also liquefied by certain bacteria, whereas agar, though a complex polysaccharide, is not subject to any such action. Thus, we have the basal media broth or bouillon, nutrient gelatin and nutrient agar, the last two being solid products of the first made by the addition of gelatin or agar, as the case may be.

In order to meet the special demands of some of the pathogenic microbes, a variety of other food materials are frequently added to the basic media. Serum, blood, glucose, glycerol and various growth factors are some of them. Other substances commonly employed in the preparation of media are egg, potatoes and milk. Quite apart from the enrichment aim, many other substances are often added to media with the object of bestowing on them a selective or differential property. Such media are called selective or differential media. Bile and bile salts, alkali, tellurite and certain dyes are some of the selective substances used. Blood also subserves the same function in addition to being an enrichment agent.

Reaction of Media. The correct adjustment of the reaction of culture media is another essential prerequisite for the successful cultivation of bacteria. Like all other living things, bacteria are very sensitive to variations in the reaction of their surroundings. Many of them require a particular narrow range of pH for optimum growth and any large deviation from this results in the suppression of growth. Small deviations, while not entirely inhibiting growth, may, nevertheless, cause considerable modification in the growth characters of bacteria. The accurate adjustment of the pH is, therefore, an important item in media preparation. Most of the pathogenic bacteria thrive best in a slightly alkaline medium of pH about 7·5 which is the same as that of the body fluids.

Phenolphthalein Method (Eyre's Method). This is an old method which has been largely discarded in favour of others which are far more accurate. Its defect is that it does not take into account the presence of several buffer substances, such as peptones, proteins, phosphates and others, in considerable quantities in the culture media. According to this method the reaction of media is expressed in what is called the Eyre's scale which indicates the number of cubic centimetres of N/1 alkali or acid required to render one litre of the medium exactly neutral to phenolphthalein. The number is expressed with the positive or negative sign prefixed to it to indicate whether the original reaction is acid or alkaline. For example, +10 signifies that 10 c.c. of N/1 NaOH have to be added to a litre of the medium to make it neutral.

Hydrogen Ion Concentration Method. This is the most accurate method for estimating the reaction of solutions. It may be done either by the electrometric or the colorimetric method. Either of these may be employed in the adjustment of the reaction, or standardisation, of culture media. The potentiometer or electrode method, though much simplified of late, is not still practicable for general laboratory use. As the colorimetric methods are simpler and at the same time sufficiently accurate, they are the ones in common use in connection with media preparation.

The symbol pH is very frequently used to express the reaction of solution and a correct idea of its significance is an essential piece of knowledge. Pure water undergoes dissociation into hydrogen and hydroxyl ions ($H_2O \rightarrow$ H-ions + OH-ions). The amount so dissociated is extremely small. The amount of hydrogen ions present in pure water is approximately 0·0000001

gram per litre. A simplified expression of this figure is 1×10^{-7}. The hydroxyl ions are equal in number to the hydrogen ions. The effect of one is neutralised by that of the other and that is why pure water is neutral in reaction. That is, the concentration of hydroxyl ions in pure water is also 1×10^{-7} which is thus the neutral point. The product of the concentration of H-ions and that of OH-ions is a constant whose value has been found to be 1×10^{-14}. It follows that when the H-ion concentration of a solution increases, as when an acid is added, the OH-ion concentration of it decreases and *vice versa*. A solution with an H-ion concentration less than 1×10^{-7}, and has, therefore, the OH-ion concentration greater than 1×10^{-7}, is alkaline, and one with more than 1×10^{-7} is acid. In an acid solution both H-ions and OH-ions are present, but the former are in excess; an alkaline solution also contains both types of ions, only the OH-ions predominate over the H-ions. The reaction level, therefore, can be expressed in terms of H-ion concentration or OH-ion concentration. But it is customary to use the former. The above is a cumbersome mode of expressing the H-ion concentration, and in order to simplify it Sorensen introduced an exponential scale denoted by the symbol pH, using the logarithm of the reciprocal of the hydrogen ion concentration. Thus $pH = \log 1/[H]$. The reciprocal of 10^{-7} is 10^{7} and $\log 10^{7} = 7$. The pH value of pure water is therefore 7. Since this is an exponential scale with 10 as base, a shift in the pH by one denotes a tenfold change in the acidity or alkalinity. Thus, a solution with pH 5 is ten times more acid than one with pH 6, and a solution with pH 9 is ten times more alkaline than one with pH 8. The neutrality point of a solution in terms of pH is 7; anything below seven is acid and above alkaline. It should be further noted that as pH depends on the reciprocal of the H-ion concentration, when the pH decreases the H-ion concentration, or acidity, increases and *vice versa*.

Buffers. The degree of dissociation occurring in any solution is profoundly influenced by the presence of substances called *buffers*. Buffering action is of special importance in the titration of media. For, all efficient culture media are buffer solutions and contain certain substances that will allow the introduction of relatively large amounts of acid or base without altering the original reaction. Buffers are thus substances that possess the capacity to resist changes in the acidity or alkalinity in solutions.

They have affinity for both H-ions and OH-ions. Excess of either, produced by the addition of acid or alkali, is immediately absorbed by the buffers and removed out from the ionised form, preventing any increase in their effective concentration. Thus, relatively large addition of acid or alkali can be made without producing appreciable changes in pH. Salts of weak acids, such as phosphates, borates, citrates, etc., behave like buffers; so also peptones, proteoses, peptides and amino acids. For example, the addition of a few drops of HCl to a litre of distilled water immediately changes the reaction from neutral to acid. But the addition of the same amount of acid to another litre of water in which a few grams of disodium phosphate have been dissolved will not produce any appreciable change in the reaction.

In the colorimetric method certain organic colouring substances, called dyes, are used as indicators. These indicator dyes are usually weak organic acids or bases which change in colour with a change in the H-ion concentration. This change in colour, however, takes place only within a definite pH range which varies for each dye and outside this range the dye is useless as an indicator. For example, phenol red solution is yellow at pH 6·8, pink at pH 7·4 and red at pH 8·4. Hence, within the pH range 6·8–8·4 alone is the phenol red useful as an indicator. The pH range of certain indicator dyes are given below.

TABLE II

The pH Range of Certain Indicator Dyes

Indicator	Range of pH	Colour change
Thymol blue ..	1·2– 2·8	Red–yellow
Bromphenol blue ..	3·0– 4·6	Yellow–blue
Bromcresol green ..	3·8– 5·4	Yellow–blue
Methyl red ..	4·4– 6·0	Red–yellow
Bromcresol purple ..	5·2– 6·8	Yellow–purple
Bromthymol blue ..	6·0– 7·6	Yellow–blue
Phenol red ..	6·8– 8·4	Yellow–red
Cresol red ..	7·2– 8·8	Yellow–red
Thymol blue ..	8·0– 9·6	Yellow–blue
Cresolpthalein ..	8·2– 9·8	Colourless–red
Phenolphthalein ..	8·5–10·5	Colourless–red
Thymolphthalein ..	9·3–10·5	Colourless–blue
B.D.H. "Universal" ..	3·0–11·0	Red–orange–yellow–green–blue–reddish violet

A series of standard solutions are made by mixing varying volumes of carefully prepared molecular solutions of an acid and its alkali salt so that they shall have definite graded H-ion concentrations, and so pH values, which are controlled by the electrode method. Usually, they are made with a pH difference of 0·2 between each. The buffer solutions are conveniently made by mixing M/15 Na_2HPO_4 and M/15 KH_2PO_4 in certain proportions. These mixtures are then enclosed in tubes in 10 c.c. amounts with 10 drops of the indicator solution, sealed and labelled. The tubes employed should be of superior quality and of uniform size. They should be scrupulously cleaned before use. For media work a pH range of 6·8–8·4 suffices and so the indicator used is phenol red in an aqueous solution with a concentration of 0·01 per cent. These tubes constitute a standard scale with reference to which media are generally standardised. Due to exposure to light the colour of the standard tubes may slowly fade in course of time when fresh tubes have to be provided.

The actual standardisation of media is carried out as follows: 2 c.c. of the medium are measured out into a thoroughly cleaned test tube and diluted with 8 c.c. of neutral distilled water. The addition of water does not materially alter the pH of the medium which is a buffer solution, while at the same time it dilutes the tint of the medium which interferes with colour matching. To the diluted medium 0·5 c.c. of the indicator is added and thoroughly mixed. A colour comparison is made against standard tubes. Depending upon whether the reaction is acid, as is usually the case, or alkaline, N/20 NaOH or N/20 HCl is added drop by drop at a time from a burette until the colour agrees with that of the standard tube with the desired pH. From the amount of the reagent added, say for example, N/20 NaOH, the total amount of N/1 NaOH that has to be added to a litre of the medium to bring it to the required pH is calculated and the same added.

It is necessary to bear in mind that media accurately standardised before sterilisation are observed frequently to have changed their pH during the process of sterilisation. Meat infusion media are especially prone to such changes. A shift to the acid side results from hydrolysis of certain constituents during heating. Experience would soon teach how to balance it by the addition

of an extra amount of NaOH during titration. A final checking may also be done with the finished product.

In the standardisation of ordinary media which have an intrinsic colour about them or are turbid the comparator method

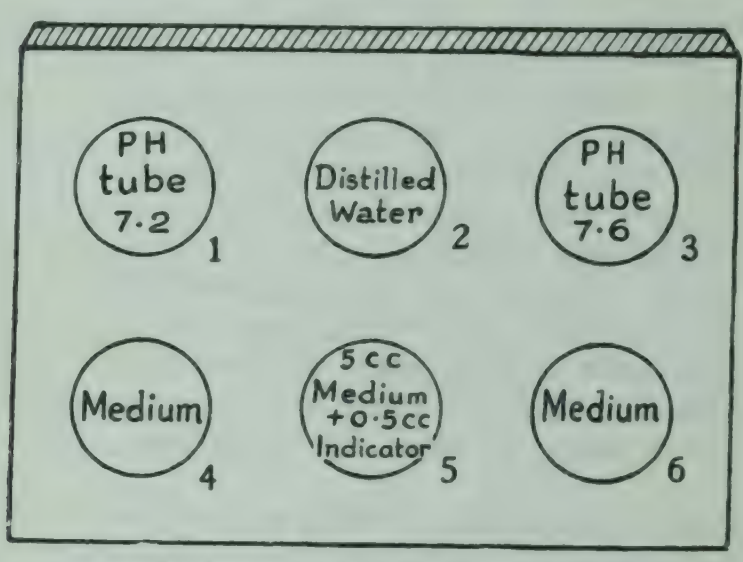


FIG. 5. Arrangement in the Comparator Method

is very helpful. In this method the matching is done in a comparator box which consists of a rack to hold two rows of three tubes each and having a ground glass at the back to disperse the light. In the figure, the extreme tubes in the back row are standard pH tubes while the middle one contains distilled water. If a pH 7·4 is required, the standard tubes employed are 7·2 and 7·6. In each of the extreme tubes of the front row 5 c.c. of the medium are placed with no indicator, while the middle one contains 5 c.c. of a 0·01 per cent. phenol red solution. A solution of N/20 NaOH is now added drop by drop from a burette to the medium-indicator mixture in the front middle tube. The volume of the NaOH solution needed to bring the colour to match with that of tube 3 in the back row is noted. From this the amount of N/1 NaOH that has to be added to a litre of the medium to bring it to pH 7·4 is calculated and added.

The standardisation of solid media like nutrient agar is more difficult than that of fluid media. It may be done in the liquid state, but accurate estimation is by no means easy. Agar of good quality seldom has any appreciable effect on the pH of the

broth with which it is mixed. Hence, only a preliminary titration of the broth base may suffice. This will do away with the difficulty that may otherwise arise from colloidal changes that may take place between the agar and the indicator if titrated after agar is added. However, the finished product can be titrated without any serious error being caused in the pH value. Cold neutral distilled water is added to melted agar and standardisation is immediately done at a temperature of 40°–45° C. before the mixture has time to jellify.

Methods of Clearing Media. A common method of clearing media is by the agency of the white of egg. This is used for media containing no coagulable proteins. The fine particles in suspension are enmeshed by the coagulating albumen which is later removed by filtration, rendering the medium clear. The white of eggs are beaten up thoroughly with a little water and added to the medium, one egg per litre. If the medium is hot, it has to be cooled to 50°–55° C. before adding the egg. The mixture is then thoroughly shaken and steamed in the Arnold steriliser for thirty to forty-five minutes. It is then removed and again shaken vigorously so as to thoroughly break up the coagulum which has been formed. It is again steamed for fifteen minutes and filtered.

Filtration. Two types of filters in common use are a special type of filter paper, Chardine filter paper, and absorbent cotton. The former is used for the filtration of fluid media. The latter is a convenient and simple material for filtering media after clearing with egg. A small piece of copper wire-gauze is placed at the bottom of a large glass funnel to prevent the cotton from jamming in the neck of the funnel. A suitably-sized square piece of cotton wool is split horizontally into two layers and their borders well trimmed. The layers are then thrust into the funnel, one layer over the other in such a way that the direction of the fibres of the one is at right angles to that of the other. The cotton is then adjusted against the sides of the funnel, if necessary, by running in a thin stream of distilled water. The funnel and filter are then warmed and the medium is poured slowly into the filter. The first portion of the filtrate may not be quite clear and require a second filtration. The filter soon becomes more efficient as the coagulum settles down, forming part of the filter, and the subsequent yield is clear. Filtration of

agar and gelatin is best done in a warm chamber and the filter should be covered with a Petri dish to prevent too rapid cooling.

Tubing of Media. After filtration, media are distributed into test tubes in appropriate quantities, about 6 c.c. for routine use. The distribution is effected by employing a large funnel with a delivery glass tube of suitable size attached to the funnel by means of a piece of rubber tube which is also provided with a thumb cock to regulate the flow. The discharging tube should be thrust deeply into the test tube before delivery is effected, so that the cotton plug area of the tube wall is not wetted with the medium. The tubes are then plugged with cotton wool and sterilised.

Sterilisation of Media. A point that should always be borne in mind in connection with the sterilisation of media is that a high temperature is injurious to the nutritive qualities of media and so exposure to excessive heat should be minimised as far as possible. Ordinary media, like nutrient broth and agar, are sterilised in the autoclave at fifteen pounds (in excess of the ordinary 15 lbs.) pressure for twenty minutes. At this pressure water boils approximately at 120° C. No doubt, this ensures certain sterilisation, but such a high temperature may also act deleteriously on the nutritive qualities of the culture media. Hence, it is usual to be satisfied with an exposure for thirty minutes at five to ten pounds pressure (about 107°–115° C.). Media which will be decomposed by this process, such as sugars, should be sterilised by the intermittent method, *i.e.* exposure to steam at 100° C. for twenty minutes for three consecutive days. In all cases where sterilisation is done in live steam, it is advisable to cover the cotton wool stopper with kraft paper to prevent drenching with steam. Media containing animal proteins, which are to be sterilised without coagulation, are sterilised by the fractional method at temperatures between 57° and 59° C. for one hour for seven or eight successive days. Temperatures above 59° C. may cause coagulation. Serum media, after inspissation, may be sterilised in the autoclave.

After sterilisation, the tubes with solid media, like agar and gelatin, are either kept upright or in a slanting position so as to obtain *slopes* which give larger surfaces. Those stored in the upright position are for use as *shake* or *stab cultures* or for making plates.

Composition of Culture Media—Infusion Broth. Fresh lean meat, preferably bovine heart, is fetched from the slaughter house, carefully freed from fat and minced very finely with the mincing machine. Five hundred grams of it are added to a litre of distilled water in a clean covered glass jar. It is allowed to infuse overnight in the ice chest. The following morning the mixture is strained through muslin, squeezing out the residue. A bright red fluid is the product, over which a thin layer of fat soon forms. With a piece of filter paper the latter is skimmed off as completely as possible. The fluid is then boiled for fifteen minutes when it becomes brown in colour and turbid owing to the changes in the contained hæmoglobin and the coagulation of dissolved proteins. It is filtered and the clear filtrate is made up to the original volume with distilled water. This constitutes meat infusion; it is a clear, light yellow fluid.

Owing to the removal of proteins by coagulation, meat infusion is poor in its nutritive properties. Hence, to improve this peptone and sodium chloride are added. Peptone is a decomposition product of proteins. The ordinary commercial peptone is really a mixture of proteoses, peptones, polypeptides and amino acids. Because of the last ingredient it is admirably adapted to serve as a nitrogen source for the growing bacteria. Peptone is soluble in water and not coagulable by heat. Thus it lends itself as a very convenient form for incorporating in media. Many types of peptones, suited for special purposes, are available in the market.

Nutrient Broth

Meat infusion	1,000 c.c.
Commercial peptone	10 g.
Sodium chloride	5 g.

These are dissolved by heat and the resulting fluid is filtered. It is acid in reaction due to the presence of sarcolactic acid. After adjusting the reaction to the desired level, it is sterilised in the autoclave. The finished product is called infusion broth, bouillon or nutrient broth. Many of the pathogenic bacteria grow freely in it.

Nutrient broth may also be prepared by substituting a 0·5—1 per cent. solution of lemco, a commercial meat extract, as the base. To this are added peptone and sodium chloride in

the above proportions, completing the preparation as in the case of infusion broth. For the growth of the more delicate pathogens, however, media made with an infusion broth are superior to meat extract medium.

Broth forms the basis of many media, solid as well as fluid. For special purposes, it is enriched with substances like serum, blood or glycerol in 5–10 per cent. and glucose in one per cent. strength. Thus we have serum broth, glucose broth and so on. Besides enhancing the nourishing qualities of media, blood also enables the detection of the hæmolytic property of bacteria and the differentiation of various strains of streptococci. Glycerinated media are usually meant for the growth of the tubercle bacillus.

Hartley's Broth. This is a digest medium. The principle of making digest media is that, instead of adding previously prepared uncoagulable, simpler and readily utilisable nitrogenous products like commercial peptone, such substances are made to form from native proteins by the action of pepsin or trypsin during the preparation of the media itself.

Ox heart or lean beef, freed from fat and minced .. .. 1,500 g.
Water .. .. 2,500 c.c.
Mix and heat in a steam steriliser to a temperature of .. .. 80° C.
Sodium carbonate (anhydrous), 0·8 per cent. solution .. .. 2,500 c.c.
Cool to 45° C. and add:
Pancreatic extract .. .. .. 50 c.c.
Chloroform .. .. 50 c.c.
Incubate the mixture at 37° C. for six hours, stirring frequently.
After digestion is completed, add 40 c.c. of pure strong hydrochloric acid, steam for 30 minutes and filter.
Adjust the reaction so as to be neutral to phenolphthalein (pH 8·4) and steam for one hour.
Filter while hot and allow to cool.
Adjust the reaction to the desired point.
Distribute in proper containers and sterilise.

If it is to be stored, filtration after the last steaming is not necessary. But add 0·25 per cent. chloroform, shake well and preserve in a cool dark place.

Hartley's broth is a very useful medium for general use. It is also widely used for the production of diphtheria toxin.

Nutrient Gelatin. Gelatin is a protein prepared from certain animal tissues, such as skin, ligaments and bones. In the dried state it is sold as sheet gelatin. It is not soluble in cold water but readily dissolves in boiling water.

Gelatin (fine quality)	100–150 g.
Broth	1,000 c.c.

The mixture is steamed for 30 minutes to effect solution. Its reaction is rendered neutral to phenolphthalein. The medium is then filtered and cleared with egg white as in the preparation of agar. It is filtered again and the pH is now adjusted to 7·6. The finished product is sterilised by the fractional method, as prolonged heating at 100° C. or autoclaving will interfere with the property of jellyfication. On cooling gelatin sets into a perfectly transparent jelly.

As gelatin melts at 24° C., it is not of use in making solid media for growing the pathogenic bacteria which require the incubator temperature for optimum growth. Hence, it is no more used for routine work. An important use of gelatin is for the study of the gelatin-digesting property of bacteria. This can be done by growing the organism at the incubator temperature and then cooling the tube down to less than 24° C. This is not quite a satisfactory method.

Nutrient Agar. Agar-agar is of vegetable origin and is derived from certain varieties of seaweeds (*Gelidium carneum*, *Gelidium cartilagenium*) found in the Chinese, Japanese and Malayan waters. It is sold in the market as dry shreads or fibres and in powder form. Both vary considerably in quality and contain many impurities. When soaked in water agar swells up into a gelatinous mass which on heating melts into a clear fluid. The solidifying property of agar depends upon its pectin content. Agar is a complex carbohydrate, and, with very rare exceptions, bacteria do not attack it.

Agar melts completely only at 98° C., but when melted it remains fluid till it is cooled down to 42° C. This property enables the incorporation into it of substances, like serum and blood, which may coagulate above 60° C. Melted agar is perfectly clear and on cooling forms a transparent jelly.

Agar fibre	20 g.
Peptone	20 g.
Sodium chloride	5 g.
Broth	1,000 c.c.

These are mixed and steamed for one to two hours to dissolve the agar. The mixture is then cleared with egg and filtered. Thereafter it is tubed or bottled in bulk as desired and sterilised.

Agar, like broth, may be enriched by the addition of substances like glucose, serum, blood and glycerol. As glucose is a reducing substance, glucose agar is often employed as an anaerobic medium.

Peptone Water (Dunham)

Peptone	10 g.
Sodium chloride	5 g.
Water	1,000 c.c.

The peptone is dissolved in water by heat and the salt is added. It may be necessary to filter. After distribution into suitable containers, sterilisation is done in the autoclave.

Its chief uses are for testing the production of indole and as a basis for preparing media for fermentation tests. Nutrient broth, as it may contain small amounts of muscle sugar from the meat, is not a suitable basis for preparing fermentation media. Any such basic medium should be completely free from any natural sugar. Peptone water is also widely used for the primary isolation of *V. choleræ*, but when so used its pH is adjusted to 8·4.

Sugar Medium. A variety of substances, mostly carbohydrates, are fermented by different bacteria, producing acid accompanied or not by gas. The term "sugars" is conveniently, though not correctly, employed to include all such fermentable substances. Fermentation reaction forms a valuable guide in the identification and classification of bacteria. The following are the substances commonly employed:

Monosaccharides

Pentoses:

Arabinose
Xylose
Rhamnose

Hexoses:
Glucose (dextrose)
Fructose (laevulose)
Mannose
Galactose
Disaccharides:
Saccharose (Sucrose)
Maltose
Lactose
Trehalose
Trisaccharides:
Raffinose
Polysaccharides:
Starch
Inulin
Dextrin
Glycogen

Alcohols

Trihydric:
Glycerol
Pentahydric:
Adonitol (adonite)
Hexahydric:
Mannitol (mannite)
Dulcitol (dulcite)
Sorbitol (sorbite)
Glucosides:
Salicin
Aesculin
Non-carbohydrates:
Inositol (inosite)

The fermentable substance is added to the peptone water in a concentration of 1 : 100. Usually the sugars are made up separately in 10 per cent. strength in distilled water, sterilised by the fractional method and kept ready for use. The amount of sugar solution necessary to make up the strength is added to the peptone water. In order to show acid production, any one of the indicators mentioned below is added. The medium

is then dispensed in sterile test tubes containing small inverted tubes (Durham's fermentation tubes) which serve to show gas production. Thereafter these culture tubes are sterilised in the Arnold steriliser for twenty minutes on three consecutive days. The air contained in the inverted tube is expelled during steaming; on cooling no air should remain in the tube. For the recognition of different sugar media, cotton wool plugs of different colours, or glass beads of different colours introduced into the media tubes, are employed.

Andrade's Indicator. This is prepared by adding normal sodium hydroxide solution to a 0·5 per cent. solution of acid fuchsin until the colour just becomes yellow. It is added to the medium in 1 per cent. strength. It is colourless at pH 7·2 but pink when the reaction is acid.

Other indicators that may be used are 0·2 per cent. phenol red in the proportion of 2 c.c. for 100 c.c. of the medium, 1 per cent. neutral red in the proportion of 0·25 c.c. per cent. and litmus solution (Kubel and Tiemann). The last named indicator is seldom used now as it is not a delicate detector of changes in pH.

Litmus Milk. This medium is employed as a routine for testing the fermentation of lactose and clotting of milk. On the other hand, proteolytic activity is evidenced by the digestion of milk, peptonisation and production of alkali.

Fresh milk is steamed for twenty minutes and allowed to stand undisturbed for twenty-four hours so that the cream may separate. The fat-free milk is then syphoned off and litmus is added just enough to give a fair blue tint. It is dispensed in tubes and sterilised by steaming by the intermittent method.

Buffered Glucose Broth. This is used for methyl red and Voges Proskauer tests.

Peptone	5 g.
Glucose	5 g.
Dipotassium hydrogen phosphate	5 g.
Water, distilled	800 c.c.

They are mixed and dissolved by heating. The mixture is cooled, filtered and the volume made up to a litre. It is tubed and sterilised by the fractional method for three days.

Koser's Citrate Medium

Sodium ammonium phosphate	1·5 g.
Potassium dihydrogen phosphate	1·0 g.
Magnesium sulphate	0·2 g.
Sodium citrate (crystals)	2·5 g.
Water, distilled	1,000 c.c.

The salts are dissolved in the water and the mixture is dispensed in test tubes and sterilised by autoclaving.

Inspissated Serum. Amounts of 5 c.c. of serum are placed in sterile test tubes aseptically by means of a sterile pipette. They are then laid in the serum inspissator, inspissated and sterilised by raising the temperature slowly to 75° C. and maintaining it for six hours. They are incubated for a day and the contaminated ones discarded.

Loeffler's Serum

Serum	3 parts
1 per cent. glucose broth	1 part

The mixture is distributed into sterile test tubes in 5–6 c.c. amounts, which are laid in a sloping position in the serum inspissator. The temperature is slowly raised to 75° C. and maintained for six hours. The serum coagulates into a white jelly with a light yellow tint. After this the tubes are sterilised in the Arnold steriliser for twenty minutes on three consecutive days. This is unnecessary if the serum was sterile.

Loeffler's serum is very satisfactory for the growth of the diphtheria bacillus. The characteristic morphological and staining features of this organism are best brought out by growing it in this medium.

The serum may be that of ox, sheep or horse. Blood is obtained from the abattoir. At the time of slaughter it is received from the carotid artery directly into sterile wide mouthed bottles which are covered and brought to the laboratory with the minimum shaking possible. After separating the clot from the sides of the bottle, they are left undisturbed in the ice chest. The following day the clear serum is pipetted off with all aseptic precautions. Serum, when contaminated, is filtered through Seitz filter. The serum is used at once or stored with 0·25 per cent. chloroform. In the latter case the preservative should be expelled before use by heating at 45° C.

If what is required is blood, it is received as before into sterile wide mouthed bottles containing broken pieces of glass or glass beads. The bottle is only half filled; it is then stoppered and shaken for about five minutes by which the fibrin is entangled in the glass pieces and subsequent clotting is thereby prevented. The same procedure is adopted in the laboratory when defibrinated rabbit blood is required. Blood can be easily obtained from the ear vein or by cardiac puncture. If properly done cardiac puncture is more satisfactory and safe from the point of view of sepsis. From 20–30 c.c. (about 50 c.c. per kilo of body weight) of blood may be collected at a time without causing untoward effect to the animal. Human blood is collected by simple vein puncture.

Hiss's Serum Water. This is used for the study of the fermentation reaction of delicate pathogens, like meningococci, streptococci and pneumococci, that will not grow well in ordinary sugar media. The production of acid with consequent change of colour and solidification of the medium is evidence of fermentation.

Serum is mixed with distilled water in the proportion of one to three, to which is added enough Andrade's indicator to give a 1 per cent. concentration. Horse serum may sometimes contain traces of sugar and for this reason it is not advisable to use it without testing. Peptone water may be used in place of distilled water. The sugars are added to the serum water-indicator mixture in the proportion of 1 per cent. The medium is then distributed into test tubes and sterilised by the fractional method.

Serum Agar. Nutrient agar (2 per cent.) is melted and allowed to cool to 45°–50° C. With a sterile pipette enough serum to make a 6–10 per cent. strength is added to each tube and thoroughly mixed by rotating rapidly between hands. The tubes are then laid in the sloping position and allowed to set. They may also be poured into sterile Petri dishes for plate cultures. After setting they are incubated for a day and the contaminated ones are discarded.

In an emergency serum-smeared agar may be used in place of serum agar. It is made by running over the surface of an agar slope a few drops of sterile serum and spreading it there.

Hydrocele or ascitic fluid, aseptically drawn, may be used instead of serum. They must have been tested for sterility.

For their storage 0·25 per cent. chloroform may be added as in the case of serum.

Blood Agar. This is prepared just as serum agar but substituting defibrinated blood for serum. It is tubed or plated as desired. Blood-smeared agar, made like serum-smeared agar, may be used to meet an emergency. Rabbit blood agar is a convenient and very satisfactory medium for general use. It is particularly valuable in the study and differentiation of certain haemolytic bacteria like the streptococcus and the pneumococcus.

Chocolate Agar (Boiled Blood Agar). Two per cent. digest agar is melted and cooled down to 60° C. Defibrinated rabbit blood is added to it in a concentration of 10 per cent. The tube is then immersed in boiling water for one minute and sloped or poured into Petri dish. Heat disintegrates the erythrocytes and converts the haemoglobin into haematin. This renders the medium excellent for the cultivation of certain fastidious pathogens like *H. influenzæ*.

Dorset's Egg Medium. This is used for the cultivation of the tubercle bacillus. Four fresh eggs are thoroughly washed with soap and water, dried and placed for a few minutes in spirit. Their contents are then well beaten up and mixed with 25 c.c. of distilled water. The mixture may be strained through sterile muslin in order to remove air bubbles. It is then distributed in quantities of 5–6 c.c. in sterile test tubes and inspissated in a sloped position at 75° C. After this the tubes are steamed for twenty minutes on three successive days. They are incubated for twenty-four hours and the contaminated ones discarded.

Glycerol Egg Medium. This is prepared as above, but with the addition of 5–6 per cent. of glycerol to the egg mixture. The human type may grow more luxuriantly in this medium.

Lowenstein Jensen Medium. This is also devised for growing the tubercle bacillus.

Mineral Salts Solution

Potassium dihydrogen phosphate (KH_2PO_4) ..	0·40 g.
Magnesium sulphate	0·04 g.
Magnesium citrate	0·10 g.
Asparagin	0·60 g.
Glycerol	2·0 c.c.
Distilled water	98·0 c.c.

Dissolve the ingredients by heat and then steam for two hours.

Starch Solution. Potato starch is added in the proportion of 30 grams to every 600 c.c. of the salt solution and thoroughly mixed. The mixture is heated in a water bath with constant stirring for 15–20 minutes. The paste so formed is allowed to remain in the water bath at 56° C. for one hour.

Egg Fluid. Fresh eggs are thoroughly washed in 5 per cent. soft soap solution and then placed in running water for sometime. Twenty eggs are usually sufficient for one litre of egg fluid. The eggs are broken into a sterile vessel, thoroughly mixed for about ten minutes and filtered through sterile gauze. One litre of it is added to 600 c.c. of starch-salt solution.

Malachite Green Solution. A 2 per cent. aqueous solution of malachite green is prepared. 20 c.c. of it are added to every 1,600 c.c. of the above mixture and thoroughly mixed.

The medium is now distributed in 5 c.c. volumes in test tubes or one ounce screw-capped bottles which are laid sloping in the inspissator and heated at 75° C. for thirty minutes. They are left in the inspissator overnight and are again heated the following day at 75° C. for thirty minutes.

The medium is rich green in colour. In virtue of the malachite green it acts as a selective medium, inhibiting the growth of the associated organisms, while at the same time enabling the unhampered or even improved growth of the tubercle bacillus. Usually before seeding, materials such as sputum and faeces are put through a preliminary treatment with 4 per cent. sodium hydroxide.

Potato Medium. Large potatoes are washed, peeled and again thoroughly cleaned. With a potato borer cylinders of potato, three-fourth inch diameter, are cut out and washed in running water to remove excess of starch. Each is then cut obliquely into two symmetrical halves, each of which is placed in a sterile test tube with the thick end resting at the bottom. Instead of ordinary tubes, potato tubes (Ehrlich's or Roux's) with a constriction a short distance from the bottom may be used; the portion below the constriction is to hold some water or broth which will serve to retard the drying of the potato. The tubes are filled with sterile water and steamed for thirty minutes. The water is then drained off and the tubes are sterilised as usual in the autoclave.

Glycerol potato is made as above, but using 5–6 per cent. glycerol instead of water. It is sometimes used for growing the tubercle bacillus.

Robertson's Medium. This is a convenient and satisfactory medium for the cultivation of anaerobes.

Fresh bovine heart is minced and placed in N/20 caustic soda, 500 grams to 500 c.c., and allowed to simmer for twenty minutes. By this the lactic acid is neutralised and the fluid comes to show a pH of about 7·5. The fluid is drained off and the meat pieces are partially dried by being spread on a filter paper or cloth. The meat is then introduced into sterile test tubes sufficient to occupy a depth of about 5 centimetres and infusion broth or Hartley's broth (pH 7·5) is added in quantities sufficient to cover the meat and occupy an extra depth of a couple of centimetres. Tne tubes are then sterilised by autoclaving. Some workers add also a vaseline seal, but this is not necessary. Others regard it advantageous to keep the medium before use in boiling water for half an hour to drive off any dissovled oxygen.

Several selective or differential media, both fluid and solid, have been elaborated for the isolation and study of the intestinal group of organisms. Their great value, especially in dealing with materials like faeces, is obvious.

MacConkey's Medium

Peptone	20 g.
Sodium taurocholate (commercial)	5 g.
Water	1,000 c.c.

The peptone is dissolved by heat. To the resulting fluid 20–25 grams of agar are added and dissolved in the steamer. The medium is cleared with egg white and filtered. Freshly prepared 1 per cent. aqueous solution of neutral red is added to it just enough (about 0·6 per cent.) to impart a distinct reddish brown colour to the medium. A rose pink colour on the addition of neutral red indicates an acid reaction; it should be corrected by adding caustic soda solution until the colour becomes reddish brown. A more accurate method is to adjust the medium previously to pH 7·6. Finally 1 per cent. lactose is added and the finished product is distributed in stoppered bottles or flasks in 100 c.c. quantities and sterilised by the intermittent method. When necessary, the medium is melted, poured in sterile plates and allowed to set.

MacConkey's medium is a very useful one for the isolation of the intestinal pathogens. In virtue of the lactose and the indicator present in it, the lactose fermenters are differentiated from the non-lactose-fermenters: the former, like *Bact. coli*, grow on it in pink colonies, whereas the latter, like *Bact. typhosum*, produce white or colourless colonies. Another advantage is that the bile salt inhibits most of the saprophytic species that may be present in the specimen.

D.E.C. Medium. This is a selective medium for the isolation of dysentery, enteric and cholera bacilli. It is a modification of Difco S S agar recently prepared by the Difco Laboratories for the isolation of dysentery bacilli (*Ind. Med. Gazette*, 1943, **78**, 43).

Lemco	0·50 g.
Peptone	0·50 g.
Sodium taurocholate	0·85 g.
Sodium citrate	0·80 g.
Sodium thiosulphate	0·85 g.
Sodium phosphate	0·75 g.
Ferric citrate	0·30 g.
Lactose	1·25 g.
Agar	2·50 g.
Neutral red, 0·25 per cent. aqueous solution	1·50 c.c.
Water	100 c.c.

A stock sterilised mixture of lemco, peptone, bile salts and plain agar at pH 7·0 is prepared and kept ready. To 100 c.c. of this mixture are added the other ingredients in the requisite quantities. The pH is adjusted to 7·4 and the medium is then sterilised by boiling for two minutes. It is now ready for pouring into plates. The medium is very clear and retains its usefulness for about a week after it is prepared and poured into plates.

Wilson and Blair's Bismuth Sulphite Medium

Nutrient agar, 3 per cent.	100 c.c.
Stock bismuth sulphite-phosphate-glucose mixture	20 c.c.
Iron-citrate-brilliant green solution	4·5 c.c.

The stock bismuth sulphite-phosphate-glucose mixture is prepared as follows: 30 grams of bismuth-anmonia-citrate scales are dissolved in 250 c.c. of boiling distilled water; 100 grams

of anhydrous sodium sulphite are dissolved by boiling in 500 c.c. of distilled water. The two solutions are mixed and while the mixture is boiling 100 grams of sodium phosphate crystals (Na_2HPO_4, $12H_2O$) are added. After cooling, 250 c.c. of a 20 per cent. glucose solution in distilled water are added. This mixture will keep for months.

The iron-citrate-brilliant green solution is made by mixing 200 c.c. of a 1 per cent. solution of ferric citrate scales in distilled water with 25 c.c. of a 1 per cent. solution of brilliant green in distilled water. This mixture also will keep for months.

This medium is used for the isolation of the enteric group of organisms. They reduce the sulphite to sulphide in the presence of glucose, giving a black colour to their colonies. The colon bacilli are inhibited by the brilliant green and by bismuth sulphite in the presence of an excess of sodium sulphite.

Dieudonne's Medium. This is an alkaline blood agar medium employed for the isolation of *V. choleræ*.

Equal parts of defibrinated ox blood and normal caustic soda solution are mixed and heated in the Arnold steriliser for 30 minutes. Three parts of this mixture are added to seven parts of 3 per cent. agar and thoroughly mixed. The medium is then plated and kept in the incubator overnight half open. After this the medium is ready for use. The finished product has a pH of 9·0–9·6. This high alkalinity gives the selective property to the medium.

Aronson's Medium. This is another selective medium for the isolation of *V. choleræ*. They grow in large red colonies in 15–20 hours. The high alkalinity of the medium inhibits the growth of other organisms and the sugars give the differentiating value.

Water	1,000 c.c.
Agar	35 g.
Meat extract	10 g.
Peptone	10 g.
NaCl	5 g.
Na_2CO_3 (10 per cent.)	60 c.c.
Sucrose (20 per cent.)	50 c.c.
Glucose (20 per cent.)	50 c.c.
Basic fuchsin (saturated)	4 c.c.
Sodium sulphite (10 per cent.)	20 c.c.

The agar is added to the water and soaked overnight. The meat extract, peptone and sodium chloride are now added and the mixture is heated in the steam steriliser for four to five hours. It is allowed to stand for some time and the supernatant fluid is distributed into suitable Erlenmeyer flasks in 100 c.c. quantities. The other ingredients, except sodium sulphite, are separately made and sterilised in the Arnold steriliser for thirty minutes. The sodium sulphite solution is sterilised by boiling several times. To 100 c.c. of the agar mixture are added six c.c. of the 10 per cent. sodium carbonate and the mixture is heated for 15 minutes at 100° C. While hot, the following are added: 5 c.c. of the sucrose solution, 5 c.c. of the glucose solution, 0·4 c.c. of the fuchsin solution and 2 c.c. of the sodium sulphite solution. The mixture is allowed to stand to permit the coarser particles to settle. Plates are poured with the clear supernatant fluid. They are allowed to set and then dried in the incubator. They are transparent and yellowish brown in colour.

McLeod's Medium. This is a selective medium for the isolation of the diphtheria bacillus. This organism resists the action of potassium tellurite and produces greyish colonies in its presence, whereas most other contaminating organisms are suppressed. On the basis of this several selective media have been devised. These media also enable the typing of *C. diphtheriæ* (see Chapter XXVII).

Infusion broth is prepared, the sterilisation being done by filtration and not by heating. Equal parts of this broth and melted 5 per cent. agar in water are mixed. To the mixture is added 7–10 per cent. freshly drawn defibrinated rabbit blood and 0·04 per cent. of potassium tellurite. They are thoroughly mixed and heated at 75° C. for 10–15 minutes and poured into sterile Petri dishes.

Bordet-Gengou Medium. This is specially designed for growing the pertussis bacillus.

100 grams of sliced potato are added to 200 c.c. of 4 per cent. aqueous glycerine. The mixture is steamed for 30 minutes and strained through gauze. The reaction is adjusted to pH 7·0.

Next a 3 per cent. agar in this potato extract is made. To one part of this potato extract are added three parts of 0·6 per cent. saline and enough agar to make up a 3 per cent. strength. The mixture is shaken thoroughly, steamed for 30 minutes and filtered.

The potato agar is dispensed in test tubes in 2–3 c.c. quantities and sterilised by steaming. The tubes are cooled down to 55° C. and an equal volume of defibrinated blood, warmed to about 40° C., is added. They are mixed thoroughly and sloped in tubes or plates.

Liver Extract Agar. This is used for the cultivation of *Brucella* organisms. Fresh ox liver is finely minced and mixed with 500 c.c. of water; it is steamed for 1½ hours and filtered through wire gauze or coarse linen. The original volume is made up with distilled water.

Liver infusion	500 c.c.
Agar	30 g.
Peptone	10 g.
Sodium chloride	5 g.
Water	500 c.c.

The ingredients are dissolved by heat. The pH is adjusted to 7·0. The medium is then filtered and sterilised in the autoclave. The addition of gentian violet in a concentration of 1 : 200,000 will give a selective quality to the medium and facilitate the isolation by inhibiting other organisms that are likely to be present.

Smith-Noguchi Medium. This medium is designed for the growth of the pathogenic spirochaetes which are strictly anaerobic. Sterile ascitic fluid introduced into long narrow test tubes each with a piece of fresh animal tissue, preferably rabbit kidney, constitutes the medium. The column of fluid is shut off from the outside air by means of a sterile vaseline seal. The long column of fluid, the reducing action of the animal tissue and the vaseline seal all contribute to the creation of a rigid anaerobic condition, particularly in the lowermost layers of the medium.

The ascitic fluid should be clear, free from bile and of high specific gravity. Both filtration and sterilisation by heat impair its quality, rendering it unsuitable for the purpose. The tissue should be as fresh as possible. In virtue of its catalase content, it destroys any hydrogen peroxide that might be formed by the growing organism and be inimical to its growth.

All manipulations should be done with severe aseptic care. A healthy rabbit is bled to death. The kidneys are removed and cut up each into 8–10 pieces. Each piece is introduced into

a sterile test tube measuring 8 × 0·5 inch. By means of a sterile 50 c.c. bulb pipette the tubes are half filled with sterile ascitic fluid. Sterile melted vaseline is then run over the medium to a depth of about an inch. The tubes are incubated for forty-eight hours and the contaminated ones are discarded. The medium will keep for long.

Before inoculating the vaseline is melted. The inoculum is then introduced to the bottom of the tube by means of a long sterile capillary pipette provided with a rubber teat. Growth occurs in about four to ten days and is indicated by a turbidity at the bottom of the tube. Material for examination is removed with a sterile capillary pipette.

Media for the Cultivation of Leptospira. Several media have been introduced for this purpose.

Noguchi's Medium

Rabbit serum	2 parts
Ringer's solution	6 parts
Rabbit plasma, citrated	1 part
Agar, 2 per cent. (neutral)	1 part

The agar is melted and cooled down to 60° C. and added to the other ingredients. The tubes are rotated well to ensure uniform mixing. A sterile vaseline seal is provided for each tube.

Schuffner's Medium

Peptone	1·5 g.
Ringer's solution	300 c.c.
Sorensen's solution	150 c.c.
Water	1,500 c.c.

Sorensen's solution is made by mixing 72 c.c. of M/15 Na_2HPO_4 and 28 c.c. of M/15 KH_2PO_4. The peptone is dissolved in water and boiled. All the other ingredients are then added and the mixture is again boiled until the phosphates have precipitated. The product is cooled, filtered, distributed in sterile test tubes in 3 c.c. quantities and autoclaved. To each tube is added 0·3 c.c. of fresh guinea-pig serum which has been sterilised by filtration. They are then heated at 56° C. for 30 minutes and tested for sterility by a day's incubation.

Lead Acetate Agar. This is used for the detection of hydrogen sulphide produced by certain bacteria.

Nutrient agar	200 c.c.
Basic lead acetate, 10 per cent. aqueous solution	1 c.c.

The lead acetate solution is sterilised and added to the agar which has been melted and cooled down to 45°–50° C. The final strength of the salt is 0·05 per cent. The medium is distributed in sterile test tubes and allowed to solidify.

CHAPTER VI

CULTURE AND IDENTIFICATION OF MICRO-ORGANISMS

The microscopic study of the organisms present in a pathological material constitutes the first step in the identification of the offending organism. Their form, size, arrangement, motility and possession or otherwise of spore and capsule should all be carefully examined. It may be pointed out that in many cases, for instance pus or sputum, the determination of motility of the organism in the primary specimen does not carry much weight and is not therefore practised as a routine step. A knowledge of the staining property of bacteria, with particular reference to Gram and acid-fast stains, also provides valuable diagnostic data. But these by themselves are seldom adequate to establish the identity of an organism. Trustworthy differences in morphology between the several species of the same genus and sometimes even between members of different genera are often lacking. For example, differentiation between the Shiga and Flexner bacilli, the haemolytic and viridans streptococci or between the *Proteus* and the *Salmonella* organisms is not possible on grounds of morphology and staining reactions alone. It is only by a multiplicity of evidence derived from a study of the morphological and staining characters, biochemical properties, immunological reactions and pathogenicity tests can a correct diagnosis of an organism be made and its causal relation to disease proved. For this it is essential to obtain the suspected organism in pure culture. The next step after microscopy, therefore, is to seed the pathological product on appropriate culture media, incubate these under suitable temperature and other requisite environmental conditions and proceed to the isolation, or separation from one another, of the contained organisms.

Often there may be more than one pathogenic species in the same lesion, for instance bronchopneumonia, boils and gas gangrene. In specimens such as motion, sputum, throat swab and the like, in addition to the causative organism or organisms,

several types of saprophytes, native to the situation, are also present. We are confronted with the same difficulty in the examination of substances like milk and water. The necessity for separating these organisms in simple pure cultures is, therefore, quite obvious.

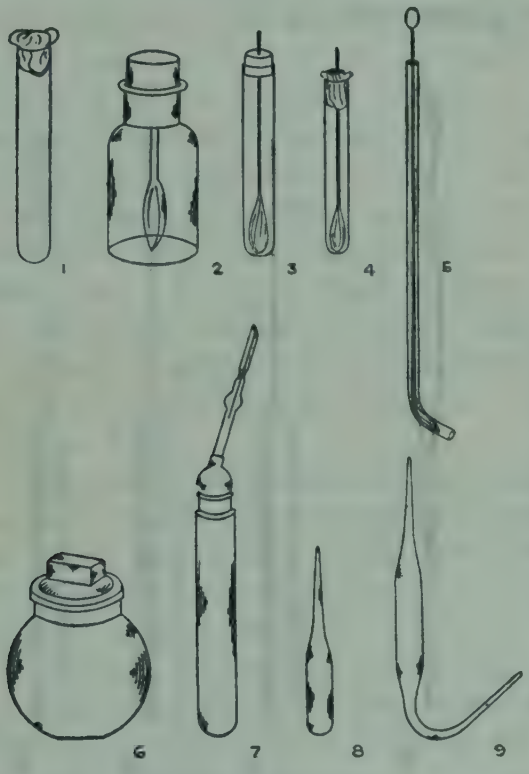


FIG. 6. **Outfits for Collecting Specimens.** 1, Sterile Test Tube ; 2, Outfit for Collecting Faeces ; 3, 4, Throat Swabs ; 5, West Swab ; 6, Sputum Bottle ; 7, Behring Venule for Collecting Blood ; 8, Ampoule; 9, Wright's Capsule for Collecting Blood.

The process of inseminating bacteria in culture media, either from pathological materials or from another culture, is termed *inoculation.* A culture grown directly from a pathological product is called a *primary culture;* cultures made from this are referred to as *subcultures.* Inoculation is done by means of a platinum wire mounted usually on a glass rod which serves as a handle. The wire should be about eight centimetres long and neither too thin nor too thick. The free end is given any shape so as to meet different requirements. Commonly, it is bent in

the form of a completely closed loop which is well fitted for the transfer of fluid cultures and for streaking on solid media. A convenient way of making it is by moulding the wire round a thin glass rod. For making stab cultures the free end is left as it is, a pointer or needle. Stouter wires may be beaten out into spatula shape for spreading or lancet head for removing sticky materials like sputum (see Fig. 7).

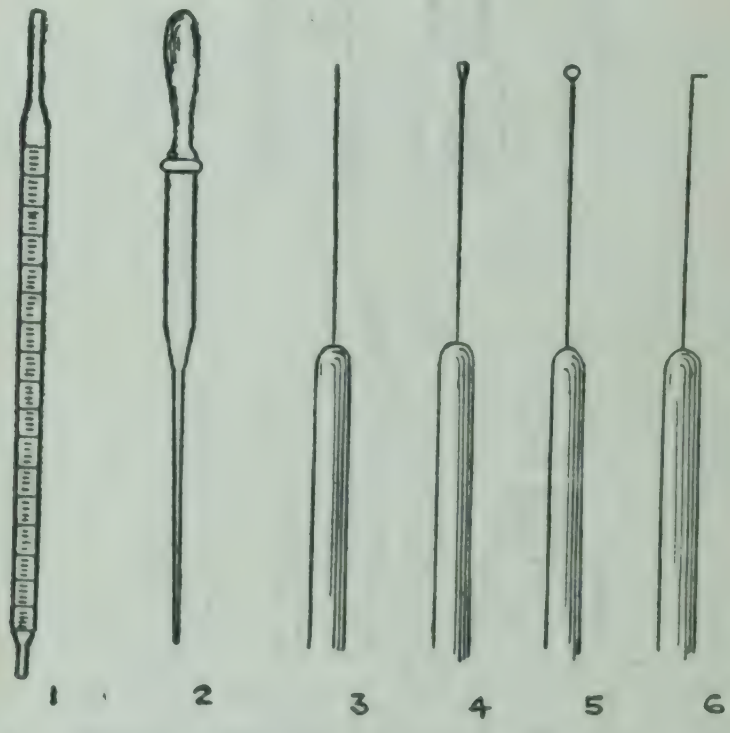


FIG. 7. 1, Graduated Pipette; 2, Pasteur Pipette; 3–6, Various Forms of Platinum Needle.

The culture tubes are held in a slanting position between the thumb and the fingers of the left hand. The cotton plugs are removed by grasping between the little finger and palm of the right hand. It is done with a slight twisting movement in order to break any possible adhesions between cotton and glass. On no account should the removed plug be placed on the bench, as such a procedure is certain to carry contamination into the tube. The platinum wire is held in the right hand. It is heated red hot, *i.e.* sterilised on a naked flame. A portion of the handle near the mount also should be sterilised. The

sterilised loop is immediately introduced into the culture tube where it is held for a few seconds to allow it to cool. This is very important, as otherwise the hot wire may kill bacteria. Allowing the loop to touch the inside wall or the top end of the medium hastens cooling. By gentle touch on the growth sufficient material is removed. The wire is then withdrawn without touching the sides of the tube. If it is a pathological product, the amount of material to be transferred is a matter of judgment based on a previous knowledge of the probable bacterial density in the material. The charged wire is then introduced into the fresh culture tube, down to the very bottom if it is a slope, and by gentle side to side strokes it is worked up the slope. Thus the inoculum is deposited on the medium in decreasing quantities towards the upper end. If it is a fluid medium, the wire is immersed in it and gently shaken or rubbed against the wall. A stab culture is made by plugging the needle right down into the medium. After inoculation the wire is withdrawn and immediately sterilised in the flame.

It is of the utmost importance that during the whole process of inoculation strict aseptic care should be exercised. The slanting position of the culture tube during inoculation prevents any direct deposit on the medium of spores or vegetative forms from the air. By this position the mouth of the test tube is also kept out of the way of the worker's expired air. Additional precautions are takan by burning the lips of the culture tubes before and after inoculation and by singeing the plug and putting out the flame with the hand.

The proper labelling and dating of the culture tubes are of supreme importance. The media after inoculation should not be left on the table but put at once in the incubator.

Isolation of Pnre Cultures. Fluid media are obviously unsuitable for the isolation of organisms. Cultivated in such a medium, the different types of organisms that may be present in any material under investigation will grow as a mixed population. Another disadvantage is that the hardy and vigorous species will outgrow the more delicate ones which are thus lost. The production of pure cultures depends upon the sowing of single cells in culture media. The early bacteriologists sought to accomplish this by dilution. They made serial dilutions of the mixed culture with sterile water or saline till at last a small portion of it was

taken to contain a single bacterium. From this, portions were subcultured in the hope that in some cases, at least, a single cell was contained in the inoculum. Obviously, the success of this method depends upon chance and so no reliance can be placed on it. The position was radically changed by the introduction of solid media. On this any material can be sown in such a way that the contained micro-organisms are scattered about as isolated individuals without any possibility of subsequent mixing. Each individual cell will multiply in course of time (eighteen to twenty-four hours) into aggregates of cells, all progeny of a single parent cell, called *colonies*. They are readily visible to the naked eye or by means of a low power hand lens. Subcultured from such isolated colonies pure cultures are obtained. The use of solid media, therefore, provides a convenient means for the isolation of pure cultures. Needless to say that all the media and materials employed for the purpose should be sterile and all manipulations should be carried out with great aseptic care.

Several methods using solid media have been evolved for the cultivation and isolation of micro-organisms in pure cultures. Plating is the commonest and most convenient one. Petri dishes are used for this. They consist of flat, round, shallow glass vessels fitted with slightly larger cover dishes. The medium is contained in a thin layer in the bottom dish, which provides a large surface. Plating is done in two ways. The culture material, any pathological specimen or mixed culture, is spread on the surface of the medium by means of a sterile bent glass rod, spatula or platinum loop—*streak plate* or *surface plate*. The spreading is done as uniformly and thinly as possible so that the organisms are sown well apart and, when they multiply, will produce discrete colonies. If the material contains numerous bacteria, a second plate and even a third may be successively inoculated with the same loop without recharging. In making surface plates an important practical point to bear in mind is that the plates should be dry. If the surface is wet, organisms, especially the motile ones, are likely to form confluent colonies, thus rendering their isolation impossible. In the other method, the specimen is mixed as evenly as possible in the melted medium contained in a test tube at 45°-50° C. and the mixture is poured into sterile plates and allowed to congeal. This is called the *pour plate method*. If the bacterial

content of the material is high, two or three serial dilutions with sterile saline or broth should be made for plating. In pouring plate care should be taken not to remove the lid and expose the bottom plate. The lid is raised at one part just enough to introduce the mouth of the test tube, which is previously sterilised by heat. The pour plate is less satisfactory than the surface plate as it involves digging of the medium for fishing out colonies. The inoculated plates are incubated in the inverted position so that the water of condensation may not wet the surface of the medium. Macroscopic colonies grow in eighteen to twenty-four hours. By means of a platinum pointer organisms from individual colonies are then carefully transferred separately into fresh media. This process is sometimes termed *fishing* of colonies. By this procedure pure cultures are obtained. Sometimes the whole process will have to be repeated with the resulting subculture in order to ensure rigid purity.

The isolation of organisms may also be accomplished by what is known as the *shake culture*. This is done in test tubes. A fairly large column of solid medium, generally glucose agar, is melted, a small quantity of the specimen is mixed well with it and then allowed to set in a vertical position. The tube is then incubated. The bacterial cells that would have been distributed apart during inoculation grow in discrete colonies in the body of the medium. The glucose functions as a reducing agent. The oxygen tension of the surface layers of such a culture medium is more or less the same as that of the atmosphere, while at the bottom the conditions are quite anaerobic. This is, therefore, a simple and fairly reliable method for the isolation of anaerobic bacteria. A preliminary process in the isolation of anaerobes is the killing of all vegetative forms by heating the specimen at 80° C. for ten to fifteen minutes, which leaves the spores unaffected. This procedure is helpful in the isolation of the sporulating anaerobes from other bacteria.

Incubation and Incubators. The growing bacteria should be provided with a temperature favourable for their development. The inoculated media are therefore placed in an incubator which maintains continuously the requisite temperature. Most of the parasitic bacteria thrive best at 37·5° C. Lower or higher temperatures are at times necessary. Suitable incubators have been constructed for providing such temperatures.

The incubator is a water-jacketed copper chamber encased in an insulating outer jacket and mounted on suitable stands. The temperature is controlled by a thermo-regulating capsule integrated into the wall of the chamber. The incubator is heated by oil lamp, gas or electricity. Incubators of various sizes are available. In large laboratories heat-regulated rooms with sections having graded temperatures may be provided.

Selective Bactericidal Substances. It has been mentioned previously that different species of bacteria show different degrees of resistance to the action of chemicals. Advantage is taken of this observation and certain bactericidal substances are employed in the isolation of organisms. Specimens are directly treated by them in appropriate dilutions for a definite time when the species that are not resistant are killed, leaving the resistant ones intact. Antiformin is a good example; it is used in the isolation of the tubercle bacillus from sputum which invariably contains several other organisms. Another chemical employed for the same purpose is sulphuric acid in 15 per cent. concentration.

Selective and Differential Media. For special purposes appropriate germicidal or bacteriostatic substances are incorporated in the culture media in which they exert a selective action on bacterial growth, favouring certain species and suppressing others. Bile salts, potassium tellurite and certain aniline dyes are in common use. If the inhibiting substance is added to a fluid medium the special organism, which is resistant to it, overgrows at the expense of the sensitive bacteria and its relative proportion to others now is far greater than in the original material. There is thus an enrichment effect. Such a fluid medium is called an enrichment medium. If, on the other hand, the substance is incorporated in a solid medium it acts selectively, favouring the development of a large number of colonies of the desired organisms than would otherwise have been possible and suppressing that of the unwanted ones. Such a solid medium is called a selective medium. Brilliant green, added to broth in a concentration of 1 in 150,000, suppresses the growth of the lactose-fermenting organisms and is used in the isolation of the typhoid and *Salmonella* organisms from stools. Bile salts inhibit the growth of intestinal saprophytes and are used in 0·5 per cent. concentration in the preparation of MacConkey's medium. Some of the selective media designed for the cultivation of the

tubercle bacillus contain certain inhibitory substances, *e.g.* crystal violet in a concentration of 1 in 10,000, which suppress the growth of associated organisms without interfering with that of the tubercle bacillus. Potassium tellurite is another useful substance and is employed for the isolation of the diphtheria bacillus. It prevents the growth of practically all other organisms found in association in throat swabs from diphtheria cases.

Certain other substances are helpful in the isolation of organisms not by inhibiting the growth of any group of organisms but by indicating the growth in some particular differentiating fashion. The addition of an indicator to a solid medium acts in this way. The presence of a fermentable sugar together with an indicator, such as litmus or neutral red, in a medium will give a red colour to the colonies of those organisms that ferment the sugar. For example, on MacConkey's medium, which contains the fermentable sugar lactose and the neutral red indicator, the coli group of organisms will grow in pink colonies due to the production of acid and consequent change in the colour of the indicator, while the non-lactose-fermenters, such as the dysentery, enteric or *Salmonella* group, will grow as colourless colonies. Blood also functions as a useful indicator. Some of the organisms do not lyse it, while others do so either partially or completely. The non-lysers do not present any change in the media. Those that partially lyse show a greenish discoloration of the medium immediately about the colonies; those that cause complete lysis leave a clear colourless ring. The diphtheria bacillus reduces tellurite and its colonies are coloured black on a tellurite plate, whereas most of the co-existing organisms are either inhibited or, if grown, not coloured.

Animal Inoculation. Another method of isolating organisms is by the inoculation of the material into suitable animals. An important application of this method is in the isolation of the tubercle bacillus from tuberculous material containing in addition contaminating organisms. These latter are killed in the animal body, whereas the tubercle bacilli produce the disease and can be recovered from the lesions in pure culture. Another instance is the recovery of a pure culture of pneumococcus from sputum by the mouse inoculation method.

Single Cell Methods. Methods have been devised to grow pure culture of bacteria from single cells. The mixed or impure

cultures are diluted and from minute droplets of these, placed under the microscope, single cells are picked out by means of special capillary pipettes and subcultured. No doubt, the ideal method for obtaining a pure culture is from a single parent cell. But the techniques so far developed are laborious and unreliable; for routine work they are of no use. Having obtained a pure culture, we proceed to establish its identity by a series of appropriate tests.

Anaerobic Cultivation of Bacteria. Molecular oxygen prevents the growth of some bacterial species, the anaerobic bacteria, while it has no such action on others. The cause of this sensitivity of the anaerobic organisms to free oxygen is not quite clear. Oxygen by itself is not inimical to the growth of anaerobes. There is some proof that in the presence of free oxygen the anaerobes form certain peroxides, probably hydrogen peroxide, which are harmful to their growth. All organisms which form peroxides are not in this way inhibited or destroyed. Some of the peroxide-producing organisms also form certain catalases, peroxidases, which, by destroying the peroxides as they are formed, protect these organisms and permit their growth. The anaerobic bacteria, on the other hand, while producing peroxides do not produce such protective catalases. Hence, they do not grow in an atmosphere containing free oxygen.

Various methods have been devised for the cultivation of anaerobic bacteria. They are based either on the exclusion of oxygen from the atmosphere in the vicinity of growth or on causing the destruction of the peroxide formed during growth through the agency of fresh animal or vegetable tissues rich in catalases. In the case of the obligate anaerobes a combination of both these methods is necessary. For example, in the cultivation of *T. pallidum*, atmospheric oxygen is shut out from the Smith-Noguchi medium by introducing a vaseline seal, while any peroxide that may be formed in the medium is neutralised at once by the catalytic activity of the piece of fresh rabbit's kidney that has been added to the medium.

Exclusion of oxygen is brought about by different methods: expelling all the dissolved air of the medium and preventing any further access; exhausting the air over the growth by an air pump and replacing it with an inert gas, such as hydrogen as in Novy's apparatus or Bulloch's jar; absorption of oxygen from

the surrounding atmosphere by the alkali-pyrogallic acid mixture; introducing certain reducing systems in the medium; deoxygenation as the result of the chemical combination of oxygen and hydrogen in the presence of a catalytic agent.

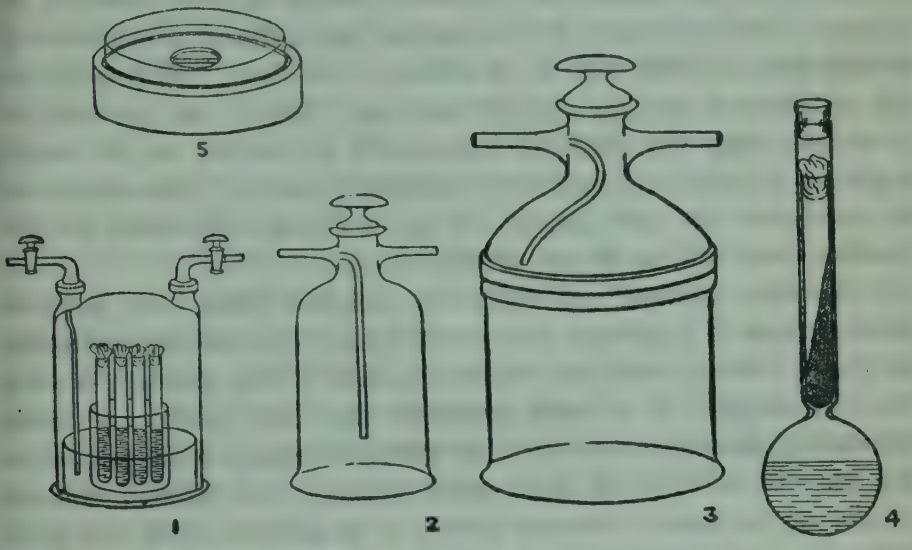


FIG. 8. **Anaerobic Apparatus.** 1, Bulloch's Jar; 2, Novy's Jar for Tubes; 3, Novy's Jar for Plates; 4, Buchner's Tube; 5, McCleod's Apparatus.

A simple method of providing anaerobic condition is by growing the organism in a deep column of solid medium in long test tubes. In virtue of its reducing capacity, glucose improves the anaerobic condition. Other reducing substances that may be used are cysteine and sodium formate. A 1 per cent. glucose agar provides the requisite gas phase for anaerobic growth, especially in its lower reaches, and is in addition very convenient. It may be used as a shake culture or stab culture. For making the former, the medium is melted and cooled down to 50° C. It is then inoculated with the material by means of a sterile capillary pipette. The contents are thoroughly mixed and suddenly cooled. For the stab culture, inoculation is done into the solid medium by means of a platinum needle. Growth is most marked in the lower portion of the medium and the number of colonies becomes fewer and fewer towards the surface, the top layers being entirely devoid of growth. For preparing pure cultures

discrete colonies from the medium are picked out and subcultured. Anaerobes that produce gas will disrupt the medium; this is a handicap. Glucose broth in deep columns contained in long tubes can also be used for anaerobic cultivation. The glucose broth tube is immersed in boiling water for five minutes to expel all the dissolved oxygen. Sterile melted vaseline or paraffin is then layered on the surface for a depth of about half to one inch and the tube is immediately cooled. This prevents the diffusion of oxygen into the medium. Before use the vaseline is melted and the medium inoculated by means of a sterile capillary pipette introduced through the vaseline. This procedure is unsuitable for the growth of gas-forming anaerobes as the vaseline seal will be blown off by the gas.

Another method of removing oxygen from the gaseous environment of a culture is by employing a mixture of pyrogallic acid and caustic soda or caustic potash. This mixture readily absorbs oxygen. It is very essential that the mixing of these substances should be effected only after enclosing the air surrounding the culture; or at least immediately after mixing, the air should be confined. Diverse pieces of apparatus, both for plate and tube cultures, have been designed employing this process of anaerobiosis: McCleod's apparatus is meant for plate culture and Buchner's tube for tube culture. This mixture is also employed to absorb the oxygen in the residual air in Bulloch's jar or Novy's apparatus after displacing the air in the jar with hydrogen. A simple method for tube culture is to push down the cotton plug to a distance of about one inch from the top of the tube, place pyrogallic acid and sodium hydroxide solution over it, close the tube at once with a tight-fitting rubber stopper or melted paraffin and then to incubate it in an inverted position.

Removal of oxygen can be effected through the agency of an aerobe. Growing in an enclosed space, the aerobe utilises all the oxygen of the contained air, rendering it suitable for the growth of anaerobes. The Petri dish method is convenient. In this two dishes of equal size are chosen. One is provided with ordinary agar and the other with a suitable medium for the anaerobe. The former is plated with the selected aerobe, e.g. the prodigiosus bacillus, and the latter with the anaerobe. Close one with the other with the edges correctly in apposition. The

final sealing is done either by slipping in a broad rubber band over the junction of the plates or by applying there a layer of plasticine. Mixing an aerobe and an anaerobe is also a convenient method of preserving culture of anaerobes in the laboratory.

Robertson's cooked meat medium is another convenient one for securing low oxygen tension. Meat contains certain reducing systems, such as unsaturated fatty acids and glutathione, which would function as efficient oxygen absorbers, producing excellent conditions for anaerobiosis. Even the exacting anaerobes will grow in it. The rigid exclusion of air from the surface is not necessary and it may be incubated aerobically. Any oxygen that may diffuse into the medium will be at once taken up by the oxidation-reduction system present in it. But, if desired, a layer of sterile vaseline or paraffin poured on the surface will prevent the access of air considerably.

Increasing the pH of a medium enhances its reducing capacity. But the anaerobic condition thus brought about is of a low degree. It may, however, be enough to permit the growth of microaerophilics and anaerobes that are not very exacting in their gas requirements.

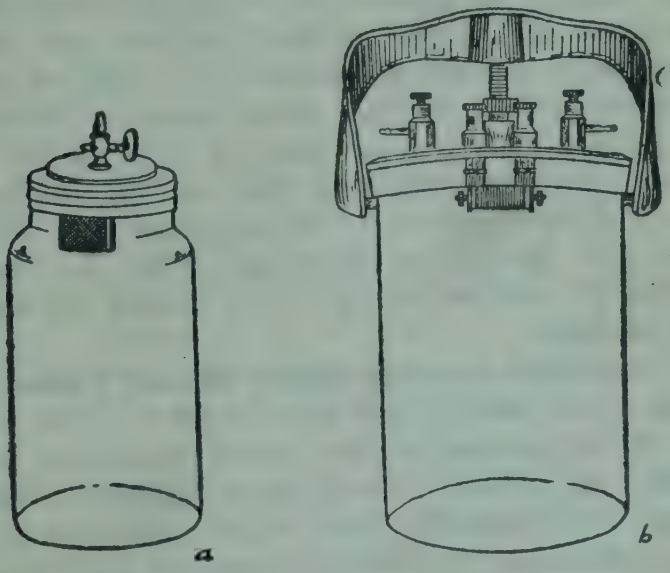


FIG. 9. *a*, McIntosh and Fildes' Jar; *b*, Improved Pattern.

Surface growth on solid media is necessary for the easy isolation of anaerobes. From this point of view none of the

methods hitherto described is suitable. Deoxygenation by the slow union of oxygen with hydrogen in the presence of a catalyst with the production of water is probably the most satisfactory method. Palladium or platinum in the spongy state and heated by the passage of an electric current functions as an efficient catalytic agent. In the McIntosh and Fildes' apparatus this is the principle employed. It consists of a jar made of stout glass or metal and provided with a close-fitting lid that can be firmly clamped down. It may be made in any suitable size. There are two valvular openings in the lid, one serves as an inlet for hydrogen and the other as an outlet for air. Asbestos is impregnated with a solution of palladium chloride and heated to drying, whereby palladium is deposited in fine black particles on the asbestos. This forms the spongy palladium. It is fastened round a small glass or porcelain spool which is suspended into the jar from the under surface of the lid. A fine resistance wire is wound round the palladiumised asbestos. Its terminals are attached to two binding screws on the top of the lid for connection with the electric supply. An electric current can thus be sent through the resistance coil and the spongy palladium heated. In order to avoid any explosion during the combustion of the oxygen-hydrogen mixture, the spool with the spongy mass and coil is enclosed in a case of fine wire-gauze. Even in spite of this safety measure, explosion may occur and, when glass jars are used, it is advisable to manipulate the apparatus inside a wooden box. Hydrogen is generated in a Kipp's apparatus. A methylene blue indicator is introduced into the jar to show the degree of anaerobiosis produced in it; with decreasing oxygen tension, the blue colour gradually vanishes.

Cultivation under Increased Carbon Dioxide Tension. Certain organisms, like *Br. abortus*, have been found to grow only in the presence of an increased carbon dioxide tension in the air in contact with the growth. Others, like the pneumococcus, gonococcus and meningococcus, grow more luxuriantly in such a gaseous environment. For the cultivation of such bacteria any anaerobic apparatus like the Bulloch's jar may be employed. The air is partially exhausted by means of a filter pump and the necessary amount of carbon dioxide is let in from a Kipp's apparatus or cylinder.

IDENTIFICATION OF MICRO-ORGANISMS

Morphology and Staining Reactions. The importance of the study of morphology and staining reactions in determining the type of an organism has been pointed out in a foregoing section. Special attention should be paid to the shape and size of the organism, its arrangement, motility and possession of spore and capsule. After a preliminary examination by the hanging-drop method, the study of the staining reactions, with particular reference to the Gram stain, is proceeded with. Incidentally, the examination of stained smears also enables to check the purity of the culture. Important as these studies are, they are inadequate for establishing the identity of an organism, except in rare instances.

Cultural Characters. The study of the morphology of colony constitutes a valuable step in the identification of species. Different bacterial species exhibit distinctive colony characters. These are usually studied on nutrient agar. The examination is best done after twenty-four hours incubation. A hand lens or a low power of a microscope enables a more critical study than the naked eye. Attention is paid to the following: nature and distribution of colonies (confluent or discrete); size; general contour—flat, raised, irregular, appearance of margin; surface smooth or rough, dry or moist; opaque, transluscent or transparent; colour; consistency; easily removed or adherent to the medium; and readily emulsifiable or not. Reference has been made above to the value of colonial appearances on special media, like blood agar, MacConkey's and blood tellurite media.

In fluid medium also bacterial growth has certain differentiating features. Points to be observed in a fluid culture are production and nature of surface growth or pellicle; the degree of turbidity and the nature of the sediment or deposit—flocculent, granular or ropy; colour and smell may also sometimes help.

Metabolic Properties. These include oxygen requirement, carbon dioxide requirement, optimum pH, optimum temperature, pigment formation, catalase production, etc. Information regarding these characters gives us additional help in determining the type of a given organism. For example, if a Gram-positive coccus produces golden yellow pigment under suitable conditions it is most likely to be *Staph. aureus;* or if an organism fails to grow

in the ordinary conditions of oxygen pressure it is one of the anaerobic group.

Biochemical Activities. Of these, the most important is the fermentation of certain carbohydrates. It has an important place in the identification and classification of bacteria. Various products, such as acid, alkali, gas and acetyl methyl carbinol, are formed during fermentation. The recognition of the presence of these products in the culture media constitutes an important means of identification. Similarly, the proteolytic activities of bacteria, especially the liquefaction of gelatin, egg and serum; the production of indole from peptone; the formation of ammonia and hydrogen sulphide; the reduction of nitrates to nitrites and the changes in litmus milk are other important biochemical activities from which considerable help can be derived in the identification of species.

Indole Production. The presence of indole is tested by adding Bohme's reagents to a peptone water growth of 24 – 48 hours' duration. One cubic centimetre of solution A is added; if no colour develops in a few seconds, the same volume of solution B is added. A positive reaction is indicated by the development of a pink to magenta colour at the interface, soon spreading to the fluid. Traces of indole can be detected by first adding a cubic centimetre of amyl alcohol, ether or xylol, shaking the mixture well and after a few seconds adding the reagents. The intensity of colour depends upon the amount of indole present which, in turn, depends upon the amount of tryptophane contained in the culture medium and so care should be taken to use a good brand of peptone, that has a high content of tryptophane, for the preparation of the medium.

Solution A:

Paradimethylamidobenzaldehyde	4 g.
Absolute alcohol	380 c.c.
Hydrochloric acid, pure	80 c.c.

Solution B:

Saturated aqueous solution of potassium persulphate.

Another method is to place a strip of sterile filter paper saturated with oxalic acid between the plug and the wall of the tube. Indole is volatile and, if it is produced, turns the paper pink.

Methyl Red Test (M.R.). This is meant for testing the production of acid. It is done by adding 3–5 drops of a 0·04 per cent. solution of methyl red to a culture in glucose phosphate medium. The development of a red colour indicates a positive reaction; the colour is yellow in negative cases.

Peptone	0·5 g.
K_2HPO_4	0·5 g.
Glucose	0·5 g.
Water	100 c.c.

(pH 7·5)

Voges-Proskauer Test (V.P.). To a two to three days old culture in the above glucose medium one cubic centimetre of a strong (15–40 per cent.) solution of potassium hydroxide is added and left at room temperature. In positive cases a chocolate colour develops slowly at the surface and may take several hours to develop fully. The test is rendered more delicate by the addition of a small amount of either creatinin or a 5 per cent. alcoholic solution of α-naphthol just before adding the alkali.

Certain organisms break down glucose to acetyl methyl carbinol. This substance in the presence of a strong alkali and atmospheric oxygen is oxidised to diacetyl which, reacting with the peptone of the medium, causes the colour change.

Nitrate Reduction. A broth culture containing 0·1 per cent. potassium nitrate is tested after three to four days' incubation.

Solution A:

α-naphthylamine	1 g.
Water	22 c.c.
Dilute acetic acid, sp.g. 1·041	180 c.c.

Dissolve the naphthylamine in water by means of heat, cool, filter and add the acetic acid.

Solution B:

Sulphanilic acid	0·5 g.
Acetic acid, dilute	150 c.c.

Add 1 ml. of solution A to the culture and then 1 ml. of solution B. A pink, red or maroon colour indicates a positive reaction, whereas absence of coloration means a negative reaction. Sometimes a negative reaction is obtained though there is reduction. This is due to the fact that the nitrites produced are still

further reduced to ammonia or even nitrogen. Cultivation in a gas fermentation tube will show up the latter.

Ammonia. Its presence is tested with a peptone water culture by adding Nessler's reagent. A brown colour develops in positive cases.

Catalase Production. This is tested by adding one ml. of H_2O_2 (10 vols.) to a 24-hour agar slope culture. The tube is held in an inclined position. In positive cases copious gas bubbles are produced.

Cholera-Red Reaction (Nitroso-Indole Reaction). Certain organisms, like *V. choleræ*, reduce nitrates to nitrites and produce indole from peptone. Hence, the addition of pure sulphuric acid to a culture of these organisms in a medium containing both peptone and nitrates causes the production of a red colour almost immediately. It is not specific to *V. choleræ;* any organism that produces both indole and nitrites will give a positive reaction. In doing the test it is important to use a brand of peptone that contains nitrate as an impurity.

Changes in Litmus Milk. The preparation of this medium has been given on page 100. Milk contains lactose and casein in the form of soluble calcium caseinogenate. If the former is fermented acid is produced, changing the colour of the indicator to red. No more changes may occur. But if considerable quantities of acid are formed, there ensues also precipitation of the caseinogen into a soft gelatinous curd (acid clot). It does not retract and alkali dissolves it completely. Change of colour to a deep blue denotes alkali production. This may happen without further changes. Or the production of alkali may be associated with the precipitation of the casein as a rennet curd, giving rise to a clear pale yellow fluid. The rennet clot is formed under the influence of bacterial enzymes. It is firm and retracts expressing a clear limpid fluid called whey. It cannot be dissolved by alkali. During coagulation by rennet the soluble calcium caseinogenate is transformed into the insoluble calcium caseinate. In the production of acid clot the acid combines with the calcium, causing the precipitation of caseinogen which is insoluble.

Production of Hydrogen Sulphide. Certain bacteria elaborate hydrogen sulphide during growth, presumably from amino acids containing sulphur, which has a differential value. It is tested by growing them in lead acetate agar (p. 110). A stab culture

s made on this medium. A black line of growth, due to the precipitation of lead sulphide, indicates the production of hydrogen sulphide.

Serological Reactions. The above mentioned characters, while throwing considerable light on the nature of the species under investigation, are not as reliable as the serological tests which are based on the antigenic structure of bacteria. These serological tests are *in vitro* antigen-antibody reactions. They depend upon the fact that bacterial infection provokes the production of specific antibodies in the host's tissues, that such antibodies are contained in the various body fluids and that these fluids, when brought into contact with the appropriate antigen, give rise to certain specific changes. Such antibodies have also been found to be produced by the tissues of certain lower animals, like the rabbit, in response to their artificial immunisation with the appropriate bacterial antigens. The immunised animals' blood will usually show a very high concentration of the specific antibodies. Especially is this the case with agglutinins. Such specific sera are spoken of as *high titre sera*. They are prepared and preserved in the laboratory to be employed for the identification of freshly isolated and unknown bacteria. Because of its simplicity and reliability the agglutination test is one of the widely used tests for this purpose. Whenever an organism is isolated from a pathological material and from its other properties suspected to be of a certain type, its identity is finally fixed by observing its bahaviour in the presence of the homologous immune serum. Taking advantage of these principles certain indirect methods of identification of the causal organism, and consequently of the diagnosis of the disease, have been developed. Well known examples of such tests are the Widal and the Wassermann; precipitation phenomenon and various skin reactions provide others.

Pathogenicity Tests. Experimental animals constitute an indispensable adjunct to the study of the pathogenic microbes. They are extensively used for testing the pathogenic property of bacteria. Not all animals, it may be remembered, are susceptible to the human pathogens. For many of the latter no susceptible animals have been discovered. On the other hand, a few of the pathogenic bacteria are common to man and some of the lower animals, e.g. *Past. pestis* and *Sp. minus*. Of the susceptible

animals, only some develop lesions identical to those produced in human infections, as for instance in tuberculosis; in certain others though the lesions may not have close resemblance in every respect, yet there are features uniformly characteristic of the particular infection, as for instance in diphtheria. In such infections as these, animal inoculation affords a very valuable, in fact the crucial, test for identification. In the case of other pathogens and the saprophytic bacteria, it has no diagnostic value, except in a negative way.

Animals are also used in the laboratory for various other purposes, such as the isolation of certain bacteria in pure culture; enchancing or attenuating the virulence or transforming one biological type to another either for experimental studies or for making biological products, *e.g.* fixed virus, vaccinia and yellow fever virus; the production and testing of immune sera and the specific test sera; conducting neutralisation tests; and the maintenance of laboratory strains of those organisms which are either only difficultly cultivable, like *T. pallidum*, or not cultivable at all, like *Sp. minus* and many filterable viruses.

The common experimental animals are guinea-pigs, rabbits, rats and mice. Monkeys are used for special purposes. Larger animals, such as horses and calves, are employed in large laboratories manufacturing biological products. Guinea-pigs are employed in the diagnosis of tuberculosis, diphtheria, anthrax, plague, leptospirosis and others. Mention may be made here that one of the commonest uses to which the animal is put is as the source of complement for the Wassermann test. Though less frequently than the guinea-pig, the rabbit also forms a common test animal. They are more prone to parasitic infections. The final differentiation of the human and the bovine types of tubercle bacilli rests on tests in rabbits. This animal is particularly useful in the diagnosis of rabies. Agglutinating and haemolytic sera are prepared from rabbits.

Methods of Inoculation. Both parenteral and alimentary routes may be employed for the introduction of the material under investigation into the animal body. The former is far more frequently chosen than the latter. Inoculation may be done in a variety of ways, the commonest being the intradermal, subcutaneous, intramuscular, intraperitoneal and intravenous. The route is usually determined by the nature of the suspected

organism and other circumstances. Great skill is required for doing intracutaneous injection successfully. A very common use of this method is for testing the virulence of *C. diphtheriæ*; 0·1–0·2 ml. is the amount usually injected. Subcutaneous and intramuscular routes are easy; either of these is used for injecting tuberculous materials. Special care must be taken to avoid the intestines when performing intraperitoneal injections. The animal is held with the head downwards, so that the intestines fall towards the diaphragm. The injection is made in the middle line in the lower third of the abdomen. At first the needle is entered subcutaneously for a short distance and then plunged at right angles through the abdominal wall into the peritoneal cavity. The intravenous method in small animals is somewhat difficult. In rabbits it is easy and the marginal ear vein is chosen for that. Injection is done in the direction of the blood flow and after dilating the vein by pressure applied in its course at the root of the ear. Swabbing with xylol or warm water may also help in dilating the veins. Inoculation may be carried out in guinea-pigs into the large superficial vein on the dorsal and inner side of the hind leg; it requires anaesthesia. In mice one of the lateral veins at the root of the tail is utilised. The animal is kept in a specially designed cylindrical cage with the tail projecting externally; the vein is dilated by immersing the tail in warm water. Other routes used in special circumstances are the intratesticular, intratracheal, intracerebral, intrathecal and intraocular. In order to introduce the test material through the respiratory route, it is sprayed into the nose or the animal is made to inhale it. In rare instances rubbing the infected material on the intact shaven skin will cause infection, for instance plague. Finally there is the alimentary route or ingestion. The material is either mixed with the animal's food or introduced into the stomach through a tube.

Great care should be exercised in the choice of the animal. It should be remembered that they are subject to natural bacterial and parasitic infections. Special attention should be paid to health, age, weight and sometimes sex. Needless to say that the site of injection should be carefully prepared; the fur is removed by shaving or by the application of a depilatory and the skin is sterilised with alcohol or tincture of iodine.

While some specimens like pus can be inoculated straight away, others will have to be prepared before injection. Sputum, motion, and the like will contain other organisms besides the main offender. Lest these associated organisms should interfere with the experiment, they should be destroyed by suitable selective methods before injection. Materials like urine and peritoneal and pleural fluids may have to be reduced in volume by centrifugation and the sediment used for injection. Infected organisms or other tissues are to be cut up into small pieces, ground up in a mortar with some coarse substances like sand, emulsified in sterile saline, allowed to stand in a test tube and the supernatant fluid used for injection. It goes without saying that all articles used should be sterile. Fluid cultures are inoculated directly, but solid cultures are first emulsified in sterile salt solution before injection. Where exotoxin is the test material, a suitable broth culture of the organism should be filtered through a bacterial filter to remove the cells and the filtrate employed for injection.

The inoculated animals are kept in carefully labelled cages made of galvanised iron wire. They should be observed every day for any abnormal condition with special reference to their general attitude, appetite, weight and temperature. The temperature is taken per rectum. The normal rectal temperature of the guinea-pig is about 101·5° and of the rabbit 101·7° F.; slight fluctuations of the temperature are normal phenomena. Animals under prolonged observation should be weighed at regular intervals as it may provide a rough indication of the progress of the disease.

Animals are usually killed with a general anaesthetic like chloroform. The disadvantage of this method is that it induces struggling, causing petichial haemorrhages in the viscera, which may obscure the true histological picture. Poisoning with coal gas is cheap and devoid of this disadvantage.

Whenever possible, post-mortem should be done immediately after death of the animal. Unavoidable delay is to be met by keeping the carcase in the cold chamber. The weight of the body should be noted before autopsy.

The carcase is soaked in 3 per cent. lysol for about ten to fifteen minutes. It is then nailed on the post-mortem board. It is good practice to have the board in a large enamelled iron

tray in order to minimise the hazard of disseminating the infectious material. The worker should protect himself with rubber gloves. It is also advisable to wear a water-proof overall. The whole operation should be done with aseptic care. It is done in the usual routine fashion. The site of inoculation is examined. After incising the skin and removing the flaps, the condition of the subcutaneous tissue and of the axillary and inguinal glands should be observed. The abdominal and thoracic cavities are exposed one after the other with different sets of sterile instruments. Gross pathology is noted as usual in an orderly way. Whether or not any special examination is necessary is determined by the particular infection under investigation. Materials for examination are removed with aseptic precautions into sterile containers or media. Peritoneal and pleural fluids are removed with sterile pipettes for culture. Similarly, blood for culture may be taken from the heart. Care should be taken that fresh sterile instruments are used every time an organ is cut into for taking materials for culture; searing of the surface before cutting into is likewise essential. Pieces of tissue are removed into fixing fluid for cutting sections.

The safe disposal of the carcase is an important step. The best way is to incinerate. Where there is no such arrangement the carcase is dipped in strong antiseptic lotion for hours and then buried deep in the earth.

CHAPTER VII

THE DESTRUCTION OF BACTERIA — STERILISATION

Sterilisation means destruction of micro-organisms. Generally, the term is used for the destruction of bacteria and their spores. The subject of sterilisation has engaged the attention of scientists from an early past. It finds practical application in many branches of human activity, such as medical science, public health work, veterinary science and industry, particularly the food industry. The modern surgical procedures are based on asepsis. Beddings, clothes and other fomites infected from patients have to be sterilised before their use for other patients. Infected materials, such as faeces, urine, pus and discharges, have to be thoroughly disinfected before disposal. The subject of bacterial destruction is of paramount importance also in the study of bacteria. One of the first essentials in practical bacteriology is the removal of all forms of life from all the materials employed in the cultivation, isolation and subsequent studies of the pathogenic microbes. Glassware, metallic apparatus, platinum needles, filtering apparatus, media and other similar articles should be freed of all germs not only before but also after their use. Infected materials and tissues, removed from patients or experimental animals for examination, have to be disinfected after use. No one method is uniformly applicable in the sterilisation of all these. Different agencies have been devised or harnessed to suit different requirements. But all have the same aim of destroying not only vegetative bacteria but also the very resistant spore forms.

Antiseptics are substances which inhibit the growth of, but do not destroy, bacterial life (Eyre). They are only bacteriostatic and when the inhibiting factors are withdrawn, the organism regains its growth activities. Disinfectants destroy bacterial life. The term germicides also means the same thing. But these terms do not imply the destruction of spores. These terms are now

not rigidly applied in their original sense but are often synonymously employed.

The exact mode of destruction is a complex process. The destructive agent acts either by the coagulation of the bacterial protoplasm, the oxidation of the protoplasm or indirectly by interfering with the process of bacterial nutrition. None of these agents can, therefore, be used for the effective destruction of the pathogenic organisms in the living tissues, as these are likewise vulnerable to the action of such agents.

The sterilising agents are of two types: physical and chemical. Bacterial filtration is only a process of mechanical removal of bacteria and does not belong to either of these groups, though the final result is the same. Antibiotics are natural antibacterial substances manufactured by certain forms of life during growth.

Physical Agents

The following physical agents, possessing bactericidal power, are of practical importance: light, heat, cold, desiccation and electricity.

Light. The value of sunlight as a sterilising agent has long been recognised. There can be little doubt that, in nature, it causes considerable disinfection. Investigations have shown that the germicidal activity of sunlight is mainly due to its invisible ultra violet component. Visible radiation is much less powerful in this respect. The invisible infra-red portion of sunlight acts by the dehydration of the bacterial cell. Moreover, the action of sunlight on the substrate is not negligible. By inducing certain changes, the latter is rendered unsuitable for bacterial growth, thus producing indirectly an antiseptic effect.

The actinic radiation is destructive not only to bacteria, but it is also lethal to all protozoal forms and to the tissue cells. The exact mode of action is still obscure. It is likely that these rays act by coagulating the colloidal protoplasm, thus rendering the continuance of life impossible. The integrity of the protein molecules constituting the protoplasm is profoundly affected by the actinic rays. It is, therefore, obvious that these rays are destructive not only to the organised cells but also to their metabolic products, such as toxins. Actinic rays have been found to destroy complement.

The penetrating power of the actinic rays is very low. Even a thin layer of glass or water can filter off much of these rays. Proteins also show the same capacity. A dark surface does not permit the passage of these rays nor does it reflect or scatter them. This shows that the action is chiefly on the surface. While surface growth is, therefore, soon destroyed, fluid cultures have to be exposed in thin layers for effective destruction. Under different conditions, the effective ultra violet content of sunlight varies widely. In the tropics, the bactericidal power of sunlight is very high. Smoke and other impurities in the atmosphere filter off much of the ultra violet content from sunlight, thus reducing its bactericidal value. Direct sunlight is more effective than diffuse sunlight.

Visible rays possess only slight germicidal potency and long exposure is necessary to produce any lethal effect. They have been shown to exhibit different degrees of activity at different wave lengths. Blue rays appear to be the most active. Certain fluorescent dyes have been found to increase the sensitivity of organisms to the action of visible rays. In the presence of such dyes, as methylene blue or eosin, these feebly effective visible rays become almost as lethal as the very energetic actinic rays. This phenomenon is called "photodynamic sensitisation". Dyes can also similarly act on certain protozoa, viruses, bacteriophage and toxins. The precise nature of the changes involved in the photodynamic activation is by no means clear. It has been explained as a final oxidation process.

Desiccation. Moisutre is absolutely necessary for the manifestation and maintenance of life. This is a general law of biology. When organisms are subjected to drying, they gradually perish. The intimate cause of death may be the denaturation of the protein colloids of the protoplasm brought about by the withdrawal of water. Dehydration may also cause interference with the enzymic activity of the cell, which may help in the process of destruction. Several factors influence the rate of death, such as the type of organism, the substrate and the method of drying.

The sensitivity to desiccation varies with the bacterial species; capsulated organisms appear to resist desiccation better than the non-capsulated bacteria. The capsule acts as a protective coating. Spores are more resistant to drying than the

vegetative forms. In their dormant state, they remain viable over very long periods.

Destruction of bacteria is not always brought about by desiccation. It also depends upon the method of desiccation employed. If bacteria are first frozen and then rapid and complete evaporation of the frozen mass is allowed to take place *in vacuo*, drying of the organism occurs with no loss of life. This method of rapid evaporation in the frozen state *in vacuo* is called the *lyophil method.* Dried in this way and preserved in the cold in sealed vacuum containers, bacteria retain their vitality and virulence over long periods. Hence, this procedure may be employed for the preservation of stock cultures. Spores too dried in this way are found to be more resistant to heat and other destructive agencies than undesiccated spores: heat does not readily coagulate the protoplasm of dried organisms. This method is also employed in the preservation of plasma, therapeutic sera and such other biological products.

Heat. Heat is a very commonly employed sterilising agent. By coagulating the cell protein it causes the death of the cell. It is used in two forms, dry heat and moist heat. The former is less powerful as a bactericidal agent than the latter owing to its relatively low power of penetration. But it has the advantage that it can be more readily employed. Each kind of heat finds particular application for sterilising particular types of materials. Manifestly, the employment of dry heat is out of question in the sterilisation of culture media just as moist heat is not convenient for the sterilisation of glassware, as these become wetted by the steam.

Dry heat destroys all vegetative bacteria at a temperature of just over 100° C. in 1½ hours. Some may be destroyed sooner. Spores are more resistant and for their complete destruction a temperature of 140° C. applied for three hours or 160° C. for one hour is necessary. Wide variations are encountered in the resistance of different species of bacteria to heat; this is also true of the spores of different species. Both the vegetative forms and spores are protected by textiles and other non-conducting materials and in such circumstances longer exposure or higher temperature is required for effective sterilisation.

Dry heat is employed in the laboratory in the form of naked flame, hot air steriliser and incinerator. Naked flame is

very handy for sterilising non-inflammable articles, like platinum loop and needle. Hot air steriliser, or hot air oven, serves for sterilising such glassware as Petri dishes, pipettes and test tubes. A temperature of 160° C. kept up for one hour is sufficient for effective sterilisation. The temperature should not be allowed to go much above 180° C., for, if it does, the cotton plugs and the paper wrappers will char, giving a smoky appearance to the glassware. Incinerator is used for the destruction of carcases or contaminated materials.

Moist heat destroys both the vegetative forms of bacteria and their spores at a much lower temperature than that with dry heat. The greater efficiency is due to its greater penetrating power. There are also other contributary reasons. A rich content of moisture renders proteins more readily coagulable and the reverse is the case when the amount of moisture is low. Illustrative of the latter fact is the greater resistance of spores. Moist heat tends to increase the water content of the bacterial protein, thereby increasing its vulnerability to heat, whereas dry heat by reducing the water content increases the resistance to heat and, therefore, raises the temperature necessary for coagulation. Also, steam is a better conductor of heat than air. The presence of small amounts of alkali or acid in the suspension fluid, too small alone to be germicidal, increases the bactericidal effect of moist heat by altering the pH. This effect is more marked with acid than with alkali. The presence of protein in the suspension fluid, on the other hand, tends to protect bacteria against heat.

Moist heat is employed in the laboratory in several ways. Substances that would perish if exposed to a high temperature are sterilised by exposing them to a lower temperature for prolonged periods. Pasteurisation of milk is an example of it. Coagulated serum and egg media are sterilised in this way. In the preparation of vaccines, killing of the organism is effected by heating the suspension or culture at 56° C. for one hour for three successive days. Boiling is a common method of sterilisation. Boiling water readily destroys all vegetative forms. Prolonged boiling destroys even the refractory spores. Sterilisation of instruments is usually done in boiling water. Steam is more efficient than boiling water. Steam at the atmospheric pressure is employed in the Arnold steriliser for sterilising

materials, like milk, carbohydrate media and gelatin, that would undergo chemical or physical changes if submitted to steam under pressure.

The material for sterilisation is submitted to circulating steam at a temperature of 100° C. for 20 minutes on three consecutive days. The first exposure kills all the vegetative forms present. Any spores that may be present will sprout into vegetative forms in the next twenty-four hours. These will be destroyed by the second steaming. If any spores still survive, the third steaming will destroy them, thus ensuring thorough sterilisation. This method is known as Tyndall's intermittent or fractional sterilisation.

Steam under pressure is more efficient than steam at atmospheric pressure. It is employed in the working of the autoclave, an apparatus devised to destroy both the vegetative forms as well as spores in one operation. Materials that are not damaged by superheated and saturated steam are sterilised by autoclaving. The destruction of unwanted cultures is also done in this way. Water boils at about 100° C. at the ordinary atmospheric pressure. If this pressure is increased, the boiling point will be likewise increased. The increase of pressure is brought about by heating steam in a closed space. This is the principle of the autoclave. When the pressure of the steam inside the closed autoclave is increased by 15 lbs. above atmospheric pressure, *i.e.* a total of 30 lbs., the temperature will rise to about 120° C. Exposure to a temperature of 120° C. for 30 minutes usually suffices to ensure thorough sterilisition. Such prolonged heating at high temperature has been shown to bring about certain vital changes in the media, rendering them unsuitable for the growth and multiplication of many of the pathogenic organisms.

Cold. It is common knowledge that cold retards putrefaction; but it has very little germicidal power. The typhoid, cholera and other organisms in culture have been found to withstand a temperature of −190° C. for 20 hours without suffering any apparent changes in their biological activities. Hence, ice and icy cold substances, like ice cream, need not necessarily be sterile. However, this resistance to cold is not a general rule. Certain bacteria, like the meningococcus and gonococcus, rapidly perish when exposed to a low temperature.

Electricity. Electricity has no direct bactericidal power. But the passage of electricity through a medium leads to some

sterilising effect. This is not due to any direct action of electricity. Heat and certain substances, like acid and hydrogen peroxide, produced during the passage of current, are responsible for this bactericidal action.

Chemical Agents

A very large number of chemical substances have germicidal property. But many of them have no practical value as germicides, as, in the concentration they are effectively bactericidal, they are also injurious to the tissue cells and, therefore, they cannot be used against infection. Again, the practical application of many of the bactericidal chemicals is contraindicated in the sterilisation of materials like culture media, food substances, contaminated linen and the like owing to the damage they cause to these materials. Notwithstanding such limitations, quite a large number of chemical agents are employed in the destruction of bacteria as well as in controlling their growth.

The mode of action of the various disinfectants varies widely. This may provide a basis for classification. Apart from their nature and concentration, several factors, such as temperature, time of action, the method of action, pH, presence of extraneous organic matter and the type and concentration of bacteria, influence greatly their activity, accelerating or retarding the velocity of reaction. A rise in temperature has an accelerating effect on the rate of reaction. Disinfection is also a function of time; the rate of sterilisation increases with the time of exposure. In general, the rate of reaction at neutrality is the lowest, increasing with the shift of pH in either direction. Another very important factor is the nature of the medium; water is an efficient solvent; it also makes the ionization of salts possible. The presence of extraneous organic matter retards the disinfectant action. A part of the disinfectant is diverted from the bacteria to the extraneous matter, thus lowering the effective concentration. The rate of reaction also depends on the kind of bacteria; for instance, the tubercle bacillus is relatively resistant to the action of hypochlorite, whereas certain others are readily destroyed in its presence. In the process of disinfection there is a close correlation of all the above mentioned factors, accelerating or retrading the velocity of the process.

Distilled Water. Distilled water is credited with some degree of disinfectant action, but there is no unanimity of opinion about this. The injurious effect it exerts may be due to the action of certain dissolved substances like traces of metals, alkali and others, and distilled water as such may not be harmful to bacterial life. Spores are not affected by distilled water. In contrast to animal and higher plant cells, the bacterial cell is insensitive to changes in osmotic pressure. Nor does it usually undergo plasmolysis or plasmoptysis by exposure to hypertonic or hypotonic solutions.

Acids. Many acids are strong germicides. Their destructive action largely depends upon the degree of electrolytic dissociation they undergo and the resulting H-ion concentration. For this reason mineral acids have greater germicidal efficiency than organic acids. The toxic action of the latter, however, does not entirely depend upon the degree of dissociation. Compared to the inorganic acids, the organic acids show low degree of ionization, and yet some of them are strongly germicidal. This is partly due to the toxic nature of the undissociated whole molecule. Some of the acids, like HNO_3, have also a specific action, probably in virtue of the anion. The resistance to acids varies considerably with different species of bacteria. *Bact. coli* has been found to survive a pH of 5 during fermentation of sugars; the tubercle bacillus withstands the action of dilute acids much longer than most of the organisms.

Alkalies. The OH-ions are mainly responsible for the disinfectant property of alkalies, but they are less active than H-ions. In general, the degree of ionization is a measure of the bactericidal potency of alkalies. Thus, KOH and NaOH are more active germicides than others, because of their higher degree of electrolytic dissociation. The kations of earthy metals possess some lethal property. This also accounts for the greater germicidal efficiency of Ba $(OH)_2$ than KOH, though the former is less ionized than the latter; to the activity of OH-ions is added the toxicity of the positive ions, producing thereby a high total germicidal effect. The germicidal effect of soap is largely dependent on the contained alkali. Some organisms are relatively resistant to the action of alkalies, as instanced by the tubercle bacillus.

Salts. Several metallic salts, particularly those of mercury, silver and copper, possess strong bactericidal properties. Per-

chloride of mercury, merthiolate, argyrol and protargol are some of the commonly employed compounds. In low concentrations salts exert a stimulating influence on bacterial growth and in high concentrations a destructive effect. Within the toxic range their germicidal activity is proportional to their concentration.

The mechanism of salt action is not clear; it may be a molecular action, ionic action or a combination of both. The kations, anions or both may be active. The ions may exert their action either through their electrical charges, through modifying influences on the solvents and independently of their electrical charges or through the specific chemical nature of the ions. In general, their sterilising capacity increases with increasing atomic weight of the metals forming salts; for example, $HgCl_2$ is more toxic than $CaCl_2$ or $MgCl_2$. Bivalent kations are, as a rule, more germicidal than monovalent kations. The presence of proteins in the dissolving fluid lowers the germicidal potency of the salts; for instance, $HgCl_2$ in watery solution is more toxic than in the presence of serum. The salts probably combine with the proteins and form insoluble proteinates, thus lowering the effective concentration of their ions in the media. Different species of bacteria evince marked variations in their susceptibility to the action of the same salt; closely allied species usually react in a similar way. The Gram-positive bacteria are generally more sensitive to the lethal action of salts than the Gram-negative ones.

The germicidal activity of certain salts depends upon their oxidising or reducing capacity. Oxidising agents, like peroxide, ozone and $KMnO_4$, are powerful disinfectants by virtue of the nascent oxygen which they readily liberate. Many of them are widely employed for disinfectant purposes. Certain reducing agents are also powerful germicides. Formaldehyde and sulphurous acid are examples of such. Formaldehyde is lethal to both vegetative forms and their spores. In the gaseous state it is frequently used for disinfecting rooms and articles for which liquid disinfectants are unsuitable. Formalin, which is 40 per cent. formaldehyde, finds extensive application as an antiseptic and preservative. It is a common preservative and fixative of tissues.

As noted above, bacteria are resistant to changes in the osmotic pressure. But salts may suppress bacterial growth

indirectly by dehydrating the protein substrate. It is this dehydrating quality that is taken advantage of in the preservation of food.

Halogens. The halogens are powerful disinfectants. Because of the relative instability of their compounds and the damage they cause to organic substrates, their practical application is not easy. Nevertheless, they are some of the widely employed disinfectants. The bactericidal property of the halogens is found to vary inversely as their atomic weight, chlorine being the most active and iodine the least.

Chlorine in the free state or combined as bleaching powder is widely employed in the purification of drinking water and swimming bath. As a germicide chlorine gas is rapidly replacing its compounds. Chloramine, eusol and Dakin's solution are some of the commonly used germicides containing chlorine. In the presence of organic matter chlorine compounds readily yield chlorine which on interaction with water liberates oxygen (nascent). Thus, as germicides chlorine and its compounds are effective in two ways: direct chlorination of the organic matter of the bacterial cell and oxidation of the cell substance.

Iodine is probably one of the most extensively used germicides. Tincture of iodine is a simple but very effective steriliser of the skin.

Alcohols and Ethers. Absolute alcohol is either not germicidal or only weakly so. But when diluted with water, it acquires marked germicidal property. A 50 per cent. dilution displays the maximum toxicity; decreasing or increasing this strength lowers the toxicity. It should be remembered that absolute alcohol markedly reduces the toxic action of other disinfectants when dissolved in it; in some cases it may be due to the inhibition of ionization of the disinfectant. This is not true always of dilute alcohol which may even enhance the antimicrobial efficiency of some disinfectants like $HgCl_2$ and $AgNO_3$. The toxicity of different alcohols towards bacteria increases with their molecular weight; methyl alcohol is thus the least germicidal. The germicidal property of ether is of a low order; it is seldom used for disinfection.

Chloroform. Chloroform in solution has strong antiseptic properties, while its vapours are actively bactericidal. For the sterilisation and preservation of sera, meant for the preparation

of culture media, chloroform is added in 0·25 per cent. concentration. It can be removed by heating at 57° C. It is also employed for preservation of media in bulk.

Glycerol. Glycerol has antiseptic properties in strong solutions—25 per cent. and above. Many viruses are not affected by 50 per cent. glycerol. In this strength it is, therefore, used for their preservation; it also destroys all the contaminating organisms. Vaccine lymph, for instance, is preserved and sterilised in this way. Glycerol is also used as a preservative of haemolytic and other stock sera.

Phenols and Cresols. These are coal-tar derivatives possessing powerful germicidal properties. Cresols, ortho, meta and para, are isomeric compounds with the same formula. Usually, they are used in a mixture called tricresol. Phenol is soluble in water, but cresols are only slightly so. The difficulty arising from low solubility is got over by saponification. The saponified cresols, when mixed with water, give rise to colloidal suspensions and are superior to phenol in their germicidal potency. Unlike salts, even in low concentrations phenols and cresols do not entirely lose their germicidal activity.

Their exact mode of action is by no means clear. It is believed to be dependent on their power of penetration in the colloidal state into the bacterial cell. Probably the proteins of the cell are coagulated forming insoluble proteinates. Before penetration, the disinfectant is adsorbed on the cell surface where a local increase in concentration is thereby brought about. This is interfered with by the presence of other suspended organic matter which, by itself adsorbing the germicide, leads to a lowering of the effective concentration in the immediate neighbourhood of the bacteria. Phenol is probably least influenced in this way.

Soap enhances the germicidal power of these disinfectants and is employed in the preparation of their emulsified products. It has been shown that the presence of NaCl or other electrolytes heightens their bactericidal effect. They are more active when freshly prepared; after a few days their activity falls, probably because of changes in their colloidal state.

They are widely employed in laboratories, in surgical work, for disinfection of rooms and for many other antiseptic purposes. Phenol is generally used as a 5 per cent. solution; in this

strength it is rapidly lethal to vegetative bacteria and somewhat less rapidly to spores. The most commonly employed products are lysol, cresol, tricresol and izal. Lysol, a saponified cresol, is about four times as active as phenol and is usually employed in a 3 per cent. solution. Phenol in 0·5 per cent. or tricresol in 0·1 per cent. strength is used in preserving vaccines and sera.

Dyes. Many of the organic dyes have disinfectant properties of a high order. Examples of these are gentian violet, crystal violet, methyl violet, brilliant green, malachite green and acriflavin. It is probable that dyes are not actually bactericidal but only bacteriostatic. Organisms vary considerably in their sensitivity to the action of dyes. Brilliant green, for example, inhibits the growth of *B. subtilis* in dilutions of 1 : 15,000,000 and staphylococci in 1 : 4,000,000, while higher concentrations, 1 : 500,000, are required to inhibit *Bact. coli* and *Bact. typhosum*. The Gram-positive microbes are, in general, more vulnerable to the action of dyes than the Gram-negative ones. A few of the dyes are useful therapeutically and are used in the treatment of wounds and skin infections. Some of the dyes are used for the preparation of selective media for inhibiting the growth of unwanted organisms without interfering with the growth of others. This selective action is also taken advantage of in the identification of bacteria.

Essential Oils. Generally, their activity is antiseptic and not disinfectant. Some of them, however, are markedly germicidal in their pure state. Vegetable and mineral oils are devoid of any antiseptic property.

Dynamics of Disinfection

During disinfection the death of bacteria does not take place all at once but in a gradual manner. In other words, it is also a function of time. The rate of killing is usually highest at the commencement and less and less as the reaction proceeds. A few organisms remain alive almost to the end. Bacteria under the influence of disinfectants are not killed in a random manner. There is some numerical relation existing between the disinfectant and the bacteria. Experimental studies indicate that in many instances the destruction of bacteria by disinfectants takes place in a logarithmic fashion. The logarithm of the number of viable bacteria at any time is proportional to the time which

has elapsed. This can be illustrated graphically. If the number of the surviving bacteria per unit volume at succeeding intervals of time are counted and the logarithms of these numbers are plotted against time, it will be found that these points tend to be in straight line. The rate of destruction of spores by disinfectants bears a similar relationship to time. The death of bacteria in old cultures also occurs in a logarithmic order.

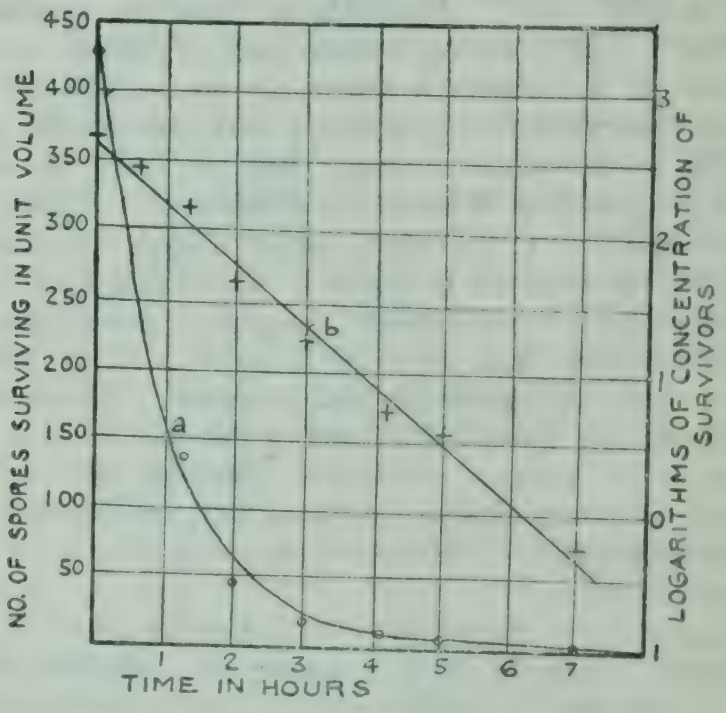


FIG. 10. **Disinfection of Anthrax Spores with 5 per cent. Phenol.** Curve *a*, Survivor time curve; Curve *b*, logarithms of concentration of survivors.

From the above data the conclusion has been reached that the process of disinfection, whether by chemical or physical agents, like moist heat, sunlight or desiccation, may be considered analogous to a chemical reaction of the monomolecular type which obeys the law of Mass Action. Indeed, in many instances, as for example the action of heavy metals on bacteria, the killing of bacteria is a chemical process. In a chemical reaction governed by the Mass law, the rate of reaction, or the reaction velocity, is proportional to the concentration of the reacting substances. In a monomolecular reaction only one of the reacting substances

is regarded as undergoing change and the velocity of the reaction is determined by its concentration. Examples of monomolecular reactions are the inversion of cane sugar by dilute acids, the decomposition of AsH_3 into As and H_2 and the disintegration of radioactive substances. In disinfection the interacting substances are the disinfectants and the bacteria. One of these, the disinfectant, is in great excess compared to the weight of the bacteria, and its concentration may, therefore, be regarded as constant throughout the reaction. Applying the rate of monomolecular reaction to disinfection, the rate of destruction of bacteria is determined by the concentration of the surviving bacteria. The number of bacteria destroyed at any given moment bears a constant proportion to the total number surviving at that time. As the number of survivals becomes less and less, the rate of disinfection becomes slower and slower until all bacteria are killed.

Although in many cases under ideal condition the killing occurs in an orderly manner with the velocity of a unimolecular reaction, in others departures from this have been observed. The death rate may be more rapid than usual in the beginning in some cases and more rapid during a subsequent period in others; it may even be quite irregular in a few cases. Bacteria are not mere masses of inert proteins and the question of variation of resistance among them cannot be ignored. As actively growing multiplying individuals, the question of age is bound to affect the inherent resistance of the individual cells to adverse agents like the disinfectants. Spores may not be materially affected by the age factor. But all these points are more of theoretical interest. What is important is to remember that disinfection is a gradual process requiring some time for its completion, during disinfection a minority of cells will survive much longer than others and that thorough sterilisation demands the destruction of all bacteria and their spores.

The concentration of the disinfectant has a profound influence on the time taken for complete sterilisation. Increase of concentration accelerates the speed of reaction. But this acceleration does not occur in simple ratios; it also differs with each disinfectant. For example, other things remaining constant, doubling the concentration of phenol increases the reaction velocity nearly 64 times, whereas doubling the strength of $HgCl_2$ only

doubles the rate of reaction, *i.e.* halves the time required for the completion of the reaction.

Temperature is another important factor which determines the rate of disinfection. Increase of temperature increases the rate fairly regularly. As the temperature of the reaction increases in arithmetical progression, the rate of disinfection increases in a geometrical ratio. For example, with a rise of temperature of 10° C. the reaction velocity of phenol is increased almost eight times, reducing the time necessary for complete sterilisation to one-eighth, and of $HgCl_2$ two to four times.

Standardisation of Disinfectants

A knowledge of the relative germicidal potency of the various disinfectants is essential in their practical application. Several methods have been devised for estimating their relative activity, but not one of them is quite satisfactory. This is not surprising in view of the fact that the process of disinfection is under the influence of several variable factors. It is imperative, therefore, that in any process of testing, every effort should be made to standardise as many of these variable factors as possible. These factors are: time of action of the germicide, age of the culture, choice of the culture medium, pH of the medium, temperature of reaction, control of resistance of test organism, specification of distinct species of organism, proportion of disinfectant to culture and the choice of a standard germicide as a control. Two methods of standardisation are now in common use: the Rideal-Walker test and the Chick-Martin test.

In the Rideal-Walker technique the germicidal power of a given disinfectant is determined in comparison with that of pure phenol under identical conditions. The ratio so obtained is called the *phenol coefficient*. The phenol coefficient of a disinfectant, therefore, tells you whether that disinfectant is weaker, equal to or stronger than phenol and in what proportion. Constant quantities of the standard organism are subjected to the action of different dilutions of phenol and of the disinfectant under test by parallel experiments. At intervals of 2½ minutes up to 15 minutes, subcultures are made into broth and incubated at 37° C. for three days. That concentration of the new disinfectant which sterilises the test culture in a given time divided by that concentration of phenol which brings about the same result in

the same time gives the phenol coefficient. The depressing effect of extraneous organic matter on the germicidal activity of disinfectants has been alluded to before. And in actual practice, it should be noted that the process of disinfection occurs not in a pure solution but in situations containing extraneous organic matter. This is one important criticism of the Rideal-Walker method. In order to meet this criticism, Chick and Martin suggested the addition of organic matter in the shape of 3 per cent. dried human faeces to the medium of action to give the reaction more naturalness. Dried yeast may replace faeces in the same concentration. Instead of varying time as in the Rideal-Walker process, a fixed time of 30 minutes is allowed for the reaction in the Chick-Martin method.

Sterilisation by Filtration

The removal of all bacterial forms from fluid materials can be accomplished by filtration through certain filtering media with pores too small to permit the passage of bacteria or spores. The great advantage of this method is that it can be used in the sterilisation of materials liable to be denatured by heat or other agencies. Hence, filtration is extensively employed in the preparation of bacteria-free toxins and in the sterilisation of serum, plasma and certain fluid media. It is also employed in the study of viruses and bacteriophage. There are several types of filters:

1. Earthenware filters: Berkefeld, Mandler and Chamberland.
2. Asbestos discs: Seitz filters.
3. Sand and paper pulp filters.
4. Collodion membranes.

The earthenware filters are made in the shape of hollow candles (Fig. 11) and the Seitz filters as flat discs. These filters are made of varying grades of permeability. The test of filtration capacity in general use is the capacity of the filter to retain a small bacterium like *Chromobacterium prodigiosum.*

Fluids under ordinary pressure do not readily pass through these filters. It is only by the application of negative or positive pressure that they can be forced through filters. Usually, filtration is effected by suction and the fluid is drawn into a conical flask of thick glass, provided with a side nozzle. The air in the flask is partially exhausted by means of an exhaust pump

attached to the side tube. Only gentle pressure (100-200 mm.), just enough to cause a satisfactory rate of filtration, should be employed: higher pressure may force into the pores minute particles and impede filtration. The pressure may have to be slightly increased in the course of filtration so as to ensure a steady rate of filtration throughout. Once begun, filtration should be finished in a reasonably short time; otherwise delicate flexible organisms, like motile spirochaetes, and very thin bacilli are apt to get through the filter, thus vitiating the filtration results.

One important drawback of the earthenware filter candles is that they are very fragile. They may have cracks, not visible to the naked eye, or imperfect joints with the metal support. The presence of these flaws should be tested before use by pressing air through the candles under water, when big air bubbles will be seen escaping through any imperfection; such candles are discarded.

Filtering candles should be cleaned every time after use. Cleaning is done by brushing with a wire brush and then boiling in distilled water. A filtration test with distilled water will reveal any clogging with organic matter, in which case heating to redness in a muffled furnace will burn off such matter and restore permeability. Trypsinisation or boiling in 2 per cent. sodium carbonate solution may also answer the purpose.

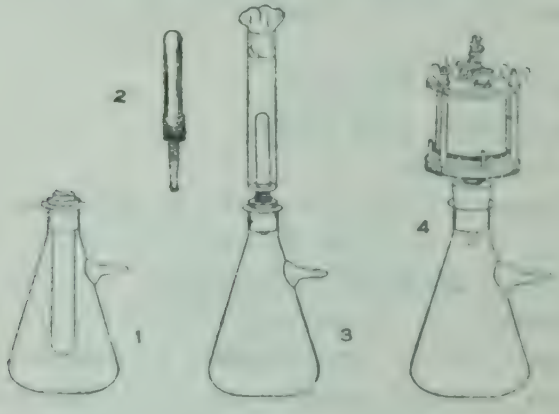


FIG. 11. **Various Types of Bacterial Filters.** 1, Chamberland; 2, Berkefeld Candle; 3, Berkefeld Filter; and 4, Seitz Filter.

Berkefeld filters (Fig. 11) are made of keiselghur or diatomaceous earth containing fossils of minute marine animals, found

in Germany and certain other regions. They are manufactured in three grades of porosity: V (Viel) coarse, N (Normal) intermediate and W (Wenig) fine; the last one has a pore size of 3 to 4 microns. Of these, V is coarse and is used for rapid clarification and for preliminary removal of most of the bacteria from liquids. The N filters are efficient bacterial filters, retaining all bacteria but permitting the passage of most of the viruses. The W candles retain all bacteria and many of the viruses.

Mandler filter is an American made earthenware filter and is a modification of the Berkefeld pattern.

The filtering candle is tightly cemented to a metal mount which is drawn out as a delivery tube. When the apparatus is assembled, the mounted candle is contained in a removable glass mantle which serves to hold the filtration fluid. Filtration takes place inward through the candle wall and the filtrate drains down through the metal delivery tube into a reservoir, usually a stout walled conical flask provided with a side exit tube. The metal holder is inserted through the rubber stopper of the flask.

The apparatus should, of course, be sterilised before use. The loosely assembled filtering portion and the receiving flask are separately sterilised either by steaming or autoclaving. The mantle is fitted up loosely to the metal holder without tightening the screw and the delivery tube is passed through the rubber stopper removed from the flask. The open end of the mantle is plugged with cotton wool and the filtering portion is wrapped in kraft paper. The flask is likewise plugged with cotton wool, and its side tube provided with cotton wool air filter; it is then wrapped in brown paper. Both parts are then sterilised. Before use, the cotton plug of the flask is replaced by the rubber stopper with the filtering part, loose connections are tightened and the side nozzle of the flask is connected by pressure tubing to a water pump or mechanical air pump. The fluid for filtration is then poured into the container and gentle negative pressure applied, which is registered by an interposed pressure gauge.

During filtration it is necessary that the column of fluid should completely surround the candle throughout so as to get the maximum amount of filtrate. To ensure this a sterile test tube, just bigger than the candle, is inverted over it so that a film of fluid will rise up between the candle and the test tube,

bathing the whole surface of the candle almost to the end of filtration. This is particularly indicated when the amount of fluid to be filtered is small. Again, when the filtration fluid is small in quantity, a sterile test tube is introduced into the filtering flask so that the filtrate can be directly collected in it instead of having to transfer it from the flask.

Chamberland filters (Fig. 11) are made of unglazed porcelain and are widely used in France. They are made of varying degrees of porosity, the designation of the common ones being Ll, Ll*a*, L2 and L3. Of these, Ll is too coarse, whereas the higher ones are similar in their filtration capacity to Berkefeld V, N and W respectively. In the small Chamberland pattern, the filtering candle itself is the container for the filtration fluid and filtration occurs from the candle outward through its wall. The candle is inserted into the flask which directly receives the filtrate.

In the Seitz pattern (Fig. 11) the actual filtering agent is an asbestos disc of special composition. It is interposed between metal holders provided with arrangements for tight joint. From a metal container the fluid passes through the disc, which is aspirated down through a delivery tube into the collecting flask. The delivery tube is inserted into the flask through a rubber stopper fitted to the flask. The filtering discs are made in different diameters, 3 c.m., 6 c.m., and 14 c.m., and in two grades of porosity: the K type for clarifying and the E K types for bacterial filtration. A filtering disc is used only once and discarded after filtration, thus solving the problem of cleaning.

Sand and paper pulp filters are very crude filters employed for removing coarse particles and also for clarifying as a preliminary step to finer filtration through bacterial filters. If such a primary filtration is not done, mucus or other gelatinous materials contained in certain specimens like nasal washings are liable to clog the fine pores of the filtering candles.

Collodion filters were first employed by Bechold (1907) in the study of colloids and he applied the term ultrafiltration to the process of differential filtration. In this, the filtering medium is a thin layer of collodion. Of all the filters, they are the least influenced by secondary factors. Membranes prepared from acetic acid collodion have a coarse structure and observations made on the size of viruses were often contradictory. In recent years, the preparation of collodion membranes was raised to a high

pitch of excellence by Elford and since then ultrafiltration has been extensively used for estimating the size of viruses and bacteriophage by observing the ability of these to pass through these membranes. The pure gel membranes are now prepared from a solution of collodion (nitro-cellulose) in acetone, to which are added varying amounts of an alcohol-ether mixture. They are called *gradocol* membranes and are made with graded degrees of porosity. Variations in permeability and pore size can be accomplished with considerable precision by suitably altering the quantity of the collodion mixture used, concentration of collodion in the mixture, ratio of solvents in the solution and the conditions of evaporation. By carefully observing these technical details, membranes with average pore size ranging from 3,000 (3 microns) to 10 millimicrons can be made. The extreme minuteness of these pores can be easily imagined when it is remembered that the size of an oxyhaemoglobin molecule is 3 to 5 and of an egg albumin molecule about 4 millimicrons.

Antibiotic Substances

It is a well recognised phenomenon that some bacteria are antagonistic to the growth of other bacteria. Investigations have revealed that this antagonistic effect is due to the activity of certain substances produced by the former group which prove inimical to the growth of the latter. Such substances have been named *antibiotic substances* or *antibiotics*. Pyocyanin has been known for long; many more have been recently discovered. An antibiotic formed by one microbial species is not necessarily toxic to the same extent to all other microbes; in fact, it may even be harmless to many. Generally, the Gram-positive bacteria are more sensitive to the action of antibiotics. As yet, we have only incomplete knowledge about their chemical structure. They belong to diverse chemical groups; some are polypeptides, others lipoids or organic bases; a few contain sulphur or a pigment group. Some have been isolated in the crystalline form. Besides being antimicrobial, they are also generally toxic to animals; a few, however, are not toxic to the tissue cells and have a high therapeutic value.

Antibiotics from Bacteria. *Pseudomonas* and *Bacillus* are two of the best known genera associated with the production of antibiotics. *Ps. pyocyanea* produces two, probably three, anti-

biotic factors (Chapter XXII). Of these, *pyocyanin*, probably the blue pigment, is a chloroform-soluble phenazine compound and *pyocyanase* appears to be a lipoid containing unsaturated fatty acids to which its antibiotic property is probably due. The third substance is an ether-soluble yellow pigment called *hemipyocyanin;* it is said to be more active towards pathogenic fungi than towards bacteria. All the three are highly toxic to higher forms of life.

Various soil microbes, such as the sporing aerobic bacilli, actinomycetes and fungi, exercise a profound antagonistic influence on the non-sporing pathogenic bacteria. That is why the latter do not survive in the soil. *B. brevis*, a Gram-positive aerobic sporulating soil bacillus, develops an antibiotic substance which has been designated *tyrothricin*. It is a polypeptide soluble in alcohol, but not in water. Tyrothricin consists of at least two components, namely *gramicidin* and *tyrocidin*. Though chemically related, their properties differ markedly. Gramicidin is highly toxic to the Gram-positive organisms, whereas tyrocidin is active against both Gram-positive and Gram-negative bacteria. How exactly gramicidin acts is not known, but tyrocidin causes lysis. Gramicidin is active both in *vivo* and *in vitro* and exhibits only low toxicity to animals. Tyrocidin is not active in *vivo* and is toxic to animals, but it appears to contribute some activity to the mixture. Several other bacteria are capable of producing similar antibiotics, but they are little studied.

Many species of *Actinomyces* have been found to develop antibiotic principles. *Actinomycin A* and *Actinomycin B* are two such substances isolated from *Actinomyces antibioticus*. They are soluble in organic solvents and have been prepared in the crystalline form. Actinomycin A is a red pigment; it is often referred to simply as actinomycin. Actinomycin A is markedly bacteriostatic and weakly bactericidal, while actinomycin B is mainly bactericidal. Both are very toxic to animals. *Act. lavendulæ* yields during growth a substance termed *streptothricin* which is soluble in water and acid alcohol but not in ether. It appears to be an organic base and contains iron. It is effective against both Gram-positive and Gram-negative organisms and resembles tyrocidin in this respect. Unlike the latter, it is of low toxicity to animals. *Streptomycin* is another similar antibiotic isolated from *Act. griseus*.

Antibiotic Substances from Moulds. Several moulds, particularly of the *Penicillium* and *Aspergillus* genera, are found to develop antibiotic substances during growth. Of these, *penicillin* is the best known and most important. It is produced by *P. notatum* and to a lesser extent by other species, such as *P. chrysogenum.* While it is highly antibacterial, it is practically non-toxic to tissue cells. It has, therefore, a high therapeutic value. It was accidentally discovered by Fleming in 1929 and was subsequently used in the isolation of organisms, like *H. influenzae*, insensitive to its action. Not until 1940 were its therapeutic possibilities begun to be thoroughly investigated. War victims provided ample field for large-scale investigation, and soon its therapeutic value in the treatment of battle wounds was established. With the first success, practical steps were taken to manufacture it on a commercial scale so as to meet the demands arising from war. The demonstration of its value in the treatment of other infections soon followed.

Penicillin is readily released into the fluid culture medium. From acidulated aqueous filtrate it is extracted with ether or amyl acetate. On shaking with dilute bicarbonate or barium hydroxide solution, the organic solvent parts with the penicillin which forms sodium or barium salt. These are then purified. In the dry state the compounds of penicillin are fairly stable. *In vitro* experiments with them provide direct evidence of the bacteriostatic effect of penicillin; bacteria under its influence become enlarged and swollen and fail to divide. Different strains of the mould yield penicillins which vary slightly in their action; these in U.S.A. have been designated F, G, X, K (Penicillin I, Penicillin II, Penicillin III, Penicillin IV respectively in England), the last being too unstable for therapeutic application. Rapid advances have been made in the isolation and purification of penicillin and in the study of its chemistry and range of usefulness.

The Gram-positive pyogenic cocci, except enterococcus, the gas gangrene anaerobes and *Cl. tetani*, the gonococcus and meningococcus, *B. anthracis*, *Actynomyces*, *C. diphtheriæ*, *T. pallidum*, *T. recurrentis* and *L. icterohaemorrhgiæ* and the virus of lymphogranuloma inguinale, are all susceptible to the action of penicillin. Definitely insensitive to its action are the members of the *Bacterium* genus, of *Proteus*, *Pseudomonas*, *Pasturella*, *Brucella* and of *Haemophilus* and also the enterococcus and *Myco. tuberculosis.*

As yet there is no standard method of assay of the potency of penicillin. Estimation of potency is now based on the degree of inhibition of growth of standard strains of staphylococci. It may be done by making comparative measurements of the turbidity of developing broth cultures under its action or, what is more common, by measuring the inhibition of growth on agar media. A tentative unit is the Oxford unit—a purely arbitrary unit defined originally as the amount of penicillin which when dissolved in 1 ml. of water gives the same inhibition (area) as a certain partially purified standard solution. For the assessment of the potency of a new preparation, the test culture is sown on an agar plate so as to yield a uniform sheet of growth. Short glass or porcelain cylinders are then pressed down in the medium. One is filled with standard penicillin solution containing one Oxford unit per cubic centimetre and the rest with varying dilutions of the new preparation. After a definite period of incubation the sizes (diameters) of the inhibition zones are measured; that caused by the standard measures about 24 mm. in diameter. From the size of the inhibition zone caused by any dilution of the new preparation the number of units per ml. in that dilution can be estimated with reference to the standard and from this the potency of the original preparation. A provisional international unit, which has been recently adopted, is defined as the activity of 0·6 micrograms of the pure crystalline sodium salt of Penicillin II or G, a quantity of which is preserved as a standard. This unit very nearly approximates to the Oxford unit.

The chemical structure of penicillin is not yet definitely known. It appears to be an organic acid, containing nitrogen in its structure. It is claimed to have been synthesised recently. Penicillin is readily soluble in water and certain organic solvents like ether. In solution it is very unstable and deteriorates rapidly, but when desiccated it keeps for long. Acids, alkalies, metallic salts, alcohols, oxidising agents and heat are all destructive to it. Penicillin, as now supplied for use, is a highly hygroscopic brown or yellow powder with the odour of decomposing grass. It is in the form of sodium or calcium salt; the latter is more stable and less hygroscopic than the former. While non-toxic to tissues, its bacteriostatic potency is of a high order; a 1 : 100 million dilution of it in the purest form hitherto obtainable inhibits the

growth of staphylococcus. Thus, it is a powerful chemotherapeutic agent. It is also said to be bactericidal. Its activity is not interfered with by the presence of pus, blood or serum, an advantage over the sulphonamides, which considerably enhance its therapeutic value. Normal acidity of the gastric juice is enough to destroy penicillin; hence oral administration is unsatisfactory. Absorption after intramuscular injection is rapid, reaching maximum concentration in blood fifteen minutes after injection; intravenous administration does not give rise to toxic effect. It can be directly injected into the meningeal, pleural and other serous cavities. Excretion is also rapid, the substance appearing in the urine soon after administration. This necessitates frequent administration of large doses in order to maintain the necessary bacteriostatic concentration in blood which is considered to be about one unit per cubic centimetre.

A total dosage of two to three million Oxford units may be necessary for the treatment of severe cases. In addition to the infections due to the Gram-positive bacteria, penicillin is very effective in the treatment of gonorrhoea and syphilis; complete cure has been claimed with relatively short but intense courses of treatment. Penicillin has also been found useful in controlling sepsis by direct application to wounds and in bacterial infections of the skin. For this purpose calcium salt is used in preference to the sodium compound, as it is more stable and less hygroscopic.

Many species of bacteria, such as *B. subtilis* and *Bact. coli*, produce a ferment, called *penicillinase*, which is destructive to penicillin. Hence solutions of penicillin when contaminated with such organisms are rapidly inactivated. This is an additional reason why rigid asepsis during the preparation of penicillin solution should be ensured.

Notatin (*penatin*, *penicillin B*) is another antibiotic produced by *P. notatum*. It is probably a flavo-protein. It is insoluble in organic solvents and is active only in the presence of glucose. It forms H_2O_2 from glucose and it is this product that is responsible for its antibiotic effect. Notatin is active against the Gram-positive as well as Gram-negative bacteria but is toxic to animals.

A number of other antibiotic metabolites, such as *penicidin*, *penicillic acid*, *patulin* and *citrinin*, have been isolated from various species of penicillium. Not much is known about their properties,

Several antibiotic principles are also produced by various species of the mould *Aspergillus*.

The exact mode of action of the antibiotic substances is not well understood, except in the case of notatin. They arrest the growth and multiplication of bacteria and *in vivo* this leads to phagocytosis. They have no significant action on bacterial toxins. The bacteriostatic activity of antibiotics far surpasses that of the sulphonamides but is less than that of the dyes. With the outstanding exception of penicillin and a few others, all the antibiotics so far known are too toxic to be used in man and animals.

Chemotherapy

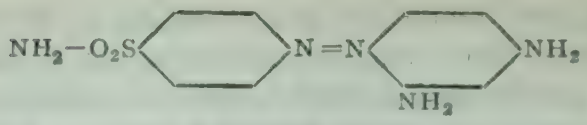
Chemotherapy, as the name indicates, is the treatment of infectious diseases with chemical agents. It seeks to cure the disease by destroying the invading parasites without causing any injury to the tissues. Prevention of infectious diseases by the use of chemical agents is also sometimes included in the term chemotherapy. The immediate aim of chemotherapy, then, is to discover such chemical agents. The most important criterion to be satisfied by them is that they should exert a maximum toxic action on the parasite and a minimum toxic action on the tissue cells of the host. In other words, between the effective parasiticidal dose and the toxic dose (toxic to the host tissue) there must be a wide range, so that the necessary concentration for the destruction of the parasite can be kept up in the body for a sufficient time without risk to the host. The destruction of the parasite, however, is not an independent action of the chemical agent. There is clear indication that the final elimination of the invading parasite is the outcome of a combined action of the drug and the defence forces of the host. Chemotherapeutic remedies may be synthetic ones, like sulphonamides, or derived from naturally occurring substances as in the case of quinine and emetine.

It was Ehrlich who first conceived the idea that it would be possible to discover or synthesise chemical agents with strong germicidal properties, which could be injected into the tissues in adequate parasiticidal concentration without causing any toxic effects. Laborious search for such a drug was then begun, culminating ultimately in the discovery of salvarsan by Ehrlich

and Hata in 1909. With this started the era of modern chemotherapy. Since then many chemotherapeutic drugs were discovered but mostly against spirochaetal and protozoan parasites. Examples of such are compounds of arsenic and bismuth for trypanosomiasis and syphilis, of antimony for Leishmaniasis and schistosomiasis and plasmochin and atebrin for malaria. At the same time, treatment of infections with coal-tar dyes also received attention, though it was not attended with much success. But it was not until recently that chemotherapy made any headway against parasitic bacteria.

The period of successful chemotherapy in bacterial infections was ushered in by the great discovery of Domagk. He found (1935) that the azo dye prontosil had marked chemotherapeutic activity in infections with haemolytic streptococci. This was followed by a phenomenal progress in this field of treatment and soon after Domagk's publication the active group of prontosil, the *p*-aminobenzene sulphonamide (sulphanilamide), was discovered. A series of allied compounds known as sulphonamides were then synthesised within a relatively short time, which were less toxic and more potent not only in streptococcal infections but also in many other bacterial diseases. To the common sulphanilamide group in these derivatives are attached different radicals which impart to the compounds different degrees of solubility, dissociation, toxicity, rate of absorption and excretion, all factors of vital importance in determining their practical value. As the result of these investigations, the clinician has now at his command some of the most remarkably effective drugs in the treatment of many serious and common bacterial diseases. Sulphonamide, sulphapyridine, sulphathiazol, sulphadiazine and sulphaguanidine are some of the best known and most useful of such substitution products; in all these, sulphanilamide is the active group.

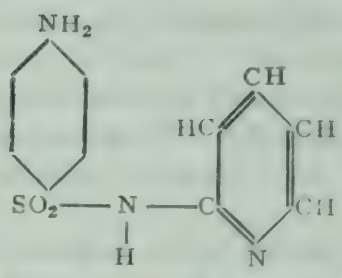
All evidence tends to show that the sulphonamides inhibit the growth of the sensitive organisms both *in vivo* and *in vitro*. Many factors influence the *in vitro* action; the concentration of the drug, quality of the medium and the number of organisms present are some of these. All compounds, with the exception of sulphaguanidine, are rapidly absorbed from the alimentary tract, chiefly from the small intestine. Absorption after parenteral therapy and excretion are likewise very rapid, making it necessary



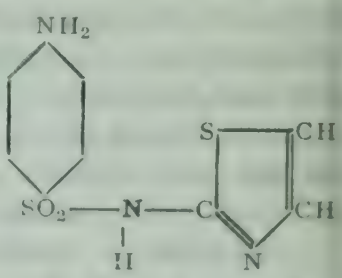
Prontosil Rubrum



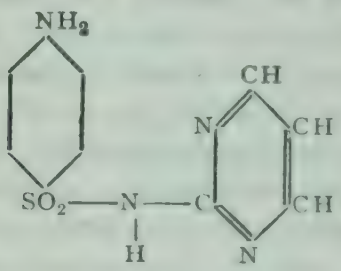
Sulphanilamide



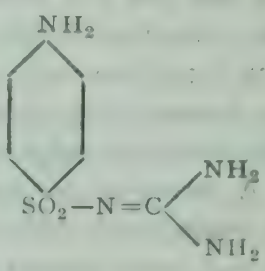
Sulphapyridine



Sulphathiazole



Sulphadiazine



Sulphaguanidine

to administer large doses at regular intervals in order to maintain the optimum bacteriostatic level (about 8–12 mg. per 100 ml.). The activity of these compounds is markedly diminished in the presence of pus. They may also give rise to toxic symptoms. Underdosage often leads to the development of resistant strains of the sensitive species.

The action of sulphanilamide is bacteriostatic and not bactericidal. The drug checks the proliferative functions of bacteria which are thus compelled to remain quiescent in the tissues. The work of destruction is then completed by the defence forces of the body, particularly through phagocytosis by the cells of the macrophage system. Sulphonamides have very little effect on bacterial toxins.

How sulphonamide brings about bacteriostasis is not clearly understood. The hypothesis of competitive inhibition has been advanced to explain the mechanism. According to it, sulphanilamide interferes with the growth of bacteria by depriving them of the use of ferments necessary for metabolism or of substances essential for the building up of the cell substance. This may be

a general mechanism applicable to the action of many other bacteriostatic substances. In order to make it clearer, it is necessary to recall certain aspects of bacterial nutrition, dealt with in the chapter on the physiology of bacteria.

Bacterial nutrition depends upon the activity of several enzymes elaborated by the bacteria. With the aid of these ferments the organism builds up from the surrounding substrate the cell substance and produces energy. It is a complex process involving the formation of several intermediate products which serve as building units. These building materials are usually synthesised by the organism itself through the intervention of enzymes. Such of these materials as are indispensable for growth, for instance certain amino acids like tryptophane, have been termed *essential metabolites* (c.f.). Any interference with the enzymic system inevitably leads to a failure of production of the concerned metabolite, resulting in the arrest of growth. Parasitism has circumscribed the enzymic system of the pathogenic bacteria and thereby lowered their synthetic powers. It is a well known fact that even among the pathogens the synthetic activity varies widely, some of them being more exacting in their food requirements than others. This is because such exacting members are lacking in such ferments as are necessary for preparing certain essential metabolites. For their growth, therefore, pre-formed metabolites have to be supplied. For example, tryptophane is essential for the building up of cell substance. By its own enzymic system *Bact. coli* is able to elaborate it from such simple nitrogen-containing substances like ammonia. In contrast to it, *Bact. typhosum*, occupying a higher position in the scale of parasitism, is often unable to synthesise tryptophane, as it is not equipped with the necessary enzymic system. Hence, for its growth tryptophane has to be supplied in the culture medium. Such essential metabolites which an organism is unable to synthesise for itself have been called *growth factors*.

It has been postulated that the action of sulphanilamide is the result of a competition of the compound with an essential metabolite, *p-aminobenzoic acid*, for the specific ferment. The close structural similarity of *p*-aminobenzoic acid and sulphanilamide favours this suggestion. When the drug is in sufficient concentration it wins in the competition, thereby depriving the organism of the use of the concerned ferment and suspending

that stage of the metabolic process. Thus, the organism is starved and growth is at a stand still. The action is, therefore, really due to a superior avidity of sulphanilamide for the ferment. There is some experimental data in support of this theory. Extracts of haemolytic streptococci or of yeast neutralise the action of sulphanilamide. This neutralising principle in yeast was found to be *p*-aminobenzoic acid. This substance or any other that is capable of yielding it on hydrolysis can *in vitro* nullify the bacteriostatic activity of sulphanilamide. Presumably, *p*-aminobenzoic acid plays an essential part in the anabolism of most bacteria, but many of them are capable of synthesising it for themselves.

The failure of culture of blood, urine and other effusions, taken when the patient is under sulphonamide therapy, is only to be expected. Sulphonamide is present in these materials in sufficient concentration to inhibit the growth of the organism and account for the failure of culture. Addition of the antagonist, *p*-aminobenzoic acid, to the culture medium is found to abolish this inhibition effect. A concentration of 5 mg. per 100 ml. of the culture medium is sufficient for this purpose. This provides an indirect proof of the antagonistic action of *p*-aminobenzoic acid.

The theory of competitive inhibition is of great significance as it offers an approach to the intimate mechanism of chemotherapy. Studies on other growth-promoting factors and their chemical analogues indicate that the theory is of wider applicability. For example, pantothenic acid is necessary for the growth of certain bacteria like *Streptococcus pyogenes* and the pneumococcus. Pentyltaurine, a sulphanilamide analogue of pantothenic acid derived by substitution of $-SO_2NH_2$ for a COOH group in the parent substance, was found to be inhibitory to these organisms. Limited experiments also tend to show that this theory may be valid even in the case of higher forms of life. Thus, it is possible to induce vitamin deficiency in experimental animals by feeding them with an analogue of the vitamin.

CHAPTER VIII

THE NORMAL FLORA OF THE HUMAN BODY

In consonance with their ubiquitous nature, micro-organisms are found everywhere on the exposed and freely accessible parts of the human and animal bodies. The study of their nature and number has received serious attention only recently. Though the type of organisms varies somewhat among communities living under different conditions of diet and climate, nevertheless, there is a remarkable constancy everywhere in the basic bacterial flora peculiar to each exposed region. For instance, *Bact. coli* is a native inhabitant of the intestinal tract and *Staph. albus* of the skin all over the world. It appears, therefore, that there are certain basic types which are not under the influence of changing conditions and others which are likely to be affected by varying conditions of diet, climate and other factors. Exotic species coming to any of these situations may find the soil uncongenial for permanent colonisation and so do not remain there for more than a transient period. The native inhabitants may also resist any such intrusion by new comers.

Many of these native microbes are harmless, but a few are potential pathogens. A sort of equilibrium has apparently been established between the latter and the host species. Normally the potential pathogens are unable to pierce the natural defence forces of the body, such as the intact skin, the mucous investment and the normal secretions, and only when there is any interference with the natural resistance in some manner do the potential pathogens invade the tissues and cause infection. Under natural conditions whether the bacterial flora exerts any beneficial role is not clear.

The nature of the native bacterial flora of any one locality depends upon many environmental factors. For example, diet and gastric acidity, which largely determines the pH of the various intestinal levels, exert a profound influence on the nature of the gastro-intestinal flora. Again, marked differences in the

normal intestinal flora are noted between the herbivorous and carnivorous animals. A low gastric acidity tends to increase the bacterial population of the gut and permit a further upward spread in the small intestine.

Alimentary Tract. Large number of organisms are ordinarily present even in a healthy mouth; they are of diverse kinds. The conditions in the mouth are very favourable for the growth and multiplication of bacteria. The remnants of food and desquamating epithelium provide the necessary food. Abnormal conditions of the gums and teeth are often present, affording suitable soil for their flourishing. There is a large influx of organisms into the mouth through food, drink and air. Against these there are at work many sterilising mechanisms, making the sojourn of such bacteria in the mouth transient. Though the saliva is only feebly bactericidal, its constant flushing action is an important factor in keeping down overpopulation of the mouth with bacteria. The abundant blood supply of the oral mucosa is also inimical to fresh bacterial colonisation. Further, there is a constant suction current towards the oesophagus, whereby organisms entering the mouth are drawn in towards the oesophagus, avoiding the surrounding walls. Organisms that are swallowed are effectively dealt with in the stomach.

The commonest organisms forming the fixed flora of the mouth are *Micrococci*, chromogenic and achromogenic; staphylococci of the albus type; streptococci, belonging to the viridans and inert types; Gram-positive bacilli, belonging to the group of aciduric bacilli or of aerobic spore-bearing bacilli; Gram-negative bacilli, including members of the coliform and *Proteus* groups; spirochaete of diverse kinds and organisms of the *Actinomyces* group.

The intestines at birth are sterile but are soon invaded by bacteria both from above and below. The intestinal flora of breast-fed infants consists largely of *Lactobacillus bifidus*. They may form about 99 per cent. of the total bacterial content in the early weeks of life, the rest being made up of enterococci and coliform bacilli. In bottle-fed infants, on the other hand, the bacterial flora is much more complex. *Lactobacillus bifidus* is uncommon; its place is taken up by the related *Lactobacillus acidophilus*, and in addition enterococci, coliform bacilli which are present in large numbers, aerobic and anaerobic spore-formers are all found in varying numbers.

In the adult, except under abnormal condition, the empty stomach is generally sterile. Most of the organisms swallowed with food and saliva are destroyed in the stomach. The survivors find the environment of the duodenum and jejunum with a pH of 5·5–6·3 very uncongenial and these regions are, therefore, practically sterile. Further down, bacteria begin to appear, and with increase in pH their number also increases till the maximum is reached in the large intestine. A few enterococci may be found in the upper part of the small intestine, but in its lower part numerous other types, like those of the coliform group and staphylococci, are also encountered. The microbial content of the large intestine consists of organisms of the colon group, enterococci, *Staphylococcus albus* and *aureus*, spore-bearing anaerobes like *Cl. welchii* and *Cl. putrificum*, aciduric bacteria such as *L. acidophilus*, thermophilic bacteria, spirochaetes and yeasts; less constant are *Proteus*, *Pseudomonos pyocyanea*, organisms of the Friedlander group and aerobic spore-formers such as *B. subtilis* and *B. mesentericus*.

Respiratory Tract. The nasal portion of the upper respiratory tract has smaller number of organisms and fewer varieties than the nasopharynx. *Staphylococcus albus* and diphtheroids are the common types seen in the nasal passages, while the green and non-haemolytic streptococci and Gram-negative cocci of the *N. pharyngis* type form the native bacterial flora of the nasopharynx and tonsils. The latter regions may contain in addition several other species, including pathogens like pneumococci, haemolytic streptococci and *H. influenzae*. But these are not so well established and their inclusion in the basic bacterial flora of these regions may not appear fully justified. The trachea, bronchi and the accessory nasal sinuses are normally sterile. Before the inspired air reaches the vocal cords, bacteria and other particulate bodies are rapidly removed from it by several agencies, such as the tortuous nasal passages, obstruction offered by hair, ciliary activity, action of mucus and disposal *via* lymphatics to the regional lymph nodes; lysozyme is also present in the nasal mucus and recently a virus inactivating agent (V1A), distinct from lysozyme, has been reported as occurring in the normal secretions of the nose. The removal of bacteria is so efficient that the expired air is almost free of bacteria.

Genito-Urinary Region. The vulva of the new born infant is sterile. The bacterial flora of the adult vulva is distinct from that of the vagina. The former consists of: pseudo-diphtheria bacilli, *Micrococcus tetragenus*, coliform bacilli, various facultative anaerobes, organisms derived from the vagina, yeasts, certain obligatory anaerobes and the smegma bacillus. The vagina of the newly born infant is sterile, but organisms make their appearance in a few hours. Save for a transient period after birth, up to the time of puberty the vaginal secretion is alkaline in reaction and a rich bacterial flora is present consisting of staphylococci, viridans and indifferent streptococci, coliform and diphtheroid bacilli and anaerobic streptococci. From the time of puberty the secretions become acid due to the glycogen present in the epithelial cells of the vaginal wall and Doderlein's bacillus permanently colonises the vagina, forming the predominant flora. After the menopause there is a reversion to the condition obtained before puberty.

In the preputial secretions of both males and females are found the saprophytic *Myco. smegmatis*. The urethral meatus of the male may contain staphylococci, diphtheroids and a short Gram-negative diplobacillus; the female urethra is either sterile or harbours a few harmless cocci.

Skin. Numerous types of organisms may be present on the surface of the skin. Some of them seem to penetrate into the sebaceous glands and make a permanent abode there. Several varieties of *Staph. albus*, *Staph. aureus*, *Sarcinae*, diphtheroids and occasionally coliform and *Proteus* bacilli constitute the characteristic bacterial flora of the skin. In addition to forming an effective mechanical barrier, the skin is also actively bactericidal and destroys any adventitious bacteria that are deposited on it.

In the skin lining the external auditory meatus are found *Staphylococcus albus*, diphtheroids and saprophytic acid-fast bacilli. Certain members of the *Pseudomonas* group are often found as permanent denizens of this region. Diphtheroids, notably *C. xerose*, are the chief inhabitants of the conjunctival sac. The constant flushing action of the tear and the destructive effect of the lysozyme content of it account for the fewness of organisms in this situation.

CHAPTER IX

THE BACTERIOLOGY OF WATER AND MILK

Water plays a dominant role in the transmission of many important intestinal infections. In order to preserve the health of a community, therefore, there is an obvious need for controlling its drinking water supply. With the increasing recognition of this fact, the question of water sanitation is acquiring greater importance. The isolation of pathogenic bacteria from water is laborious and often impracticable. Nor does a negative result of any such examination constitute a conclusive proof of the absence of such organisms. Consequently, it does not form part of the routine water analysis. What is sought for is to obtain some indirect evidence of the possible presence of pathogens in the water. Excretal pollution, as reflected by the presence of the colon bacillus in a water source, is accepted as providing this evidence. But natural waters contain a variety of micro-organisms and a brief consideration of these is necessary for a better understanding of the problem of water sanitation.

The bacterial flora of the natural waters may be conveniently classified into two groups: those that form the normal natural inhabitants of water and those that may find their way into it from extraneous sources, mainly air, soil and excreta, and make their abode of water for varying periods of time. Our knowledge about both these groups of organisms, especially of the former, is very meagre, as it is difficult to grow them in artificial culture media. The native water bacteria have been grouped as follows:

1. Higher bacteria, frequently the sheathed forms placed in the *Chlamydobacteriales* and including the sulphur and iron bacteria.
2. The *Coulobacteria*, usually occurring in lakes and other bodies of water.
3. The spirochaetes, very long forms may be encountered.
4. A variety of bacilli:
   (*a*) Fluorescent—*Pseudomonas fluorescens.*

(*b*) Chromogenic, forming different types of pigments—*Chr. prodigiosum*, *Chr. violaceum*, *Chr. aurescens*, and others.

(*c*) Non-chromogenic—members of the *Achromobacterium* group, thermophiles, aerobic spore-forming bacilli of unknown taxonomic position, and others.

5. Cocci—Chromogenic, such as *Sarcina lutea*, and non-chromogenic, such as *Micrococcus aquatilis*, *Micrococcus candicans* and others.
6. Nitrogen-fixing bacteria—*Azotobacter aquatile.*
7. Nitrifying bacteria—like *Nitrosomonas* and *Nitrobacter.*

These aquatic microbes are found in all types of natural waters, in tanks, lakes, rivers and wells. Recent studies tend to show that the bacterial populations of seawater are similar to those of other natural waters. The natural waters undergo constant fluctuations in their organic content and correlated with such changes are also encountered fluctuations of their bacterial flora.

Many of the water bacteria are difficult to grow in the laboratory media; special conditions of growth are necessary for their cultivation. For instance, the growth of the nitrifying bacteria is inhibited by the presence of organic matter. Again, the number of organisms is found to increase on preservation of water in the laboratory for a few hours. For these reasons even under the best of conditions, plate counts offer but imperfect data regarding the actual number of organisms present. But it is not so very important from the point of the potability of water, as the contaminating organisms, that are likely to be injurious on swallowing, grow with relatively less difficulty under laboratory conditions than the natural water bacteria.

Contaminating Organisms in Natural Waters. In addition to the native microbial population, diverse forms of bacteria are usually present in natural waters as contaminants from external sources. Such sources are air, soil and excreta, chiefly human.

Bacteria in the air remain attached to the dust particles, and a close correlation is said to exist between the number of bacteria found in the air and the number of suspended dust particles present in it. Hence, the heavily dust-laden atmosphere of cities contains a much larger bacterial population than the air in the

open country. Fewer organisms are present in the mountain air than in the air of the planes and practically none in the air over mid-ocean. Air is being continuously replenished with microbes from the respiratory tract, through emanations from the surfaces and through blown up dust from the soil. But it is also being constantly purified to some extent by the sun's radiations; this natural sterilising action is probably more marked in the tropics. The types of organisms present in the air may exhibit some slight variation in different localities. For example, the air in the vicinity of hospitals and dwellings may contain pathogenic organisms, like the tubercle bacilli, smallpox virus and the pyogenic cocci, and serve as a medium of transmission of infection. But such variations are too trivial to make any appreciable effect on the general bacterial flora; nor do they render the general air unwholesome. Certain organisms are commonly present in the air. They are the various species of moulds and yeasts, aerobic spore-formers, like *B. subtilis*, and various micrococci which are often pigmented. These air microbes settle down in the waters along with the anchoring dust and are also carried down by rain.

Soil is another extraneous source from which water derives its microbial contamination. It is a great repository of innumerable kinds of micro-organisms, collectively called *soil bacteria*. A gram of average field soil has been computed to contain 100-50,000 million living bacteria. They mostly reside in the upper six inches of soil; below a depth of about four to five feet organisms are scanty. These soil denizens are of diverse kinds and include the various types that are concerned with the decomposition of organic matter in the soil, the nitrifying and the nitrogen-fixing bacteria, organisms of the amylobacter group, various species of actinomyces and the anaerobic and aerobic spore-formers. A few species of the soil bacteria, like *Clostridium tetani*, the gas gangrene organisms and *Clostridium botulinum*, are potential pathogens. Soil is also being constantly contaminated with organisms from animal and human sources, particularly through excreta and decomposing infected carcases. Among these may be such important pathogenic organisms like *V. cholerae*, the dysentery bacilli, the enteric group of organisms, *Brucella* organisms, streptococci, *Past. pestis*, the anthrax bacillus and others, but most of these perish rapidly in the soil. One

exception to this is the anthrax bacillus, the spores of which are able to survive for several years. There is some dispute about the fate of the enteric and the cholera bacilli in the soil; they may remain quiescent (without multiplying) in the soil possibly up to two or three months.

The contribution of soil to the germ population of water is far more substantial than that of air. Being always in direct contact with soil, natural waters derive from it a perpetual supply of their bacterial content. Rain water flowing into them also brings in a periodic supply of bacteria from the soil. The water flowing over the surface is more prolific of bacteria and more dangerous than the percolating water as the latter undergoes natural filtration through several layers of soil. Organisms of excretal origin may gain access into the water supply in two ways; indirectly after a temporary sojourn in the soil and directly into the water. As mentioned above, they do not multiply in the soil. Contamination of water by the organisms present in the soil, particularly with those derived from the human intestines, has considerable significance from a sanitary point of view. When taken by the mouth the potential pathogens, like the tetanus and gas gangrene organisms, do not usually cause infection, but it is not so with the intestinal pathogens whose normal portal of entry is the gastro-intestinal tract. Contamination of water with the latter is very dangerous. That is why great importance is attached to the preservation of water sheds and surroundings of wells and tanks free from excretal pollution. Apart from the fact that faecal bacteria may be washed by rain water directly into natural waters, certain intestinal pathogens, like the enteric bacilli, gain access into shallow wells and reservoirs from adjoining privies, latrines and cesspools by way of ground water. It has been found that under suitable conditions faecal organisms may be carried to very great depths in the soil, 100–200 ft., by ground water.

Contamination of natural waters with excreta may also take place directly. This is the most dangerous form of pollution from the standpoint of public health. Sewage may be directly let into rivers and lakes. The habit of bathing and washing in the tanks, rivers, lakes and in other collections of surface water also leads to direct contamination of these with organisms of intestinal origin. The great danger is from pollution of water

with human excreta; pollution with animal excreta is seldom risky, as only very few infections of animals are transferable to man. Evidence of contamination of natural waters with excreta is provided by the presence of *Bact. coli*, *Cl. welchii* and *Str. faecalis*, all normal inhabitants of the intestines. The intestinal pathogens may be present only in very small numbers and it is difficult to detect them. It may also be remembered that faeces may be from healthy individuals and pathogens need not be present at all even when the water may show the presence of the normal bacterial flora of the gut.

Sewage. Sewage is a dilute solution of faecal matter and other waste products of a community. It contains plenty of complex organic matter which forms an ideal pabulum for the growth and multiplication of micro-organisms. Naturally, many organisms contained in sewage belong to the intestinal group, both normal as well as parasitic and pathogenic, such as those of the *Bact. coli* group, *Str. faecalis*, *Cl. welchii*, and probably *Bact. typhosum* and *V. cholerae*. Others belonging to the sewage proper are *Proteus vulgaris* and the anaerobic spore-bearing bacilli, like *Cl. sporogenes*. Sewage is thus an important vehicle of transmission of infection of the water-borne type and its effective and timely disposal is a pressing sanitary problem. Whatever the process of treatment of sewage, the basic action is one of bacterial activity. The complex compounds are transformed by decomposition and oxidation through various intermediate stages finally into simple compounds, like carbon dioxide, water and nitrates. In this process the pathogenic bacteria are not involved. Their survival time in water and sewage is probably less than that in soil and during disposal delicate pathogens, like the typhoid bacillus, do not multiply but tend to disappear rapidly, probably within a few days or weeks. Hence, it is obvious that fresh contamination of water with sewage is more dangerous than past contamination. It is also not safe to assume that the effluent matter after treatment is entirely free from pathogenic bacteria, unless chlorination or some such antiseptic process has been employed in the final stage of treatment.

Water for drinking purposes is obtained from various sources, all classified under two groups: surface water and deep water. Rain water derives its contamination only from the atmosphere and is probably the purest natural water. Other surface waters

are those found in rivers, lakes, tanks and shallow wells. Deep waters are those of deep wells and springs. The bacterial content of a water depends upon its type. Surface waters will contain greater variety and numbers of microbes than deep waters; for, the former being on the surface are subject to direct and heavy contamination, whereas the latter, in their passage to the underground strata, have undergone natural filtration through several layers of soil, after which the chances of gross pollution are few.

Several physical and biological factors are likewise concerned in determining the bacterial content of waters. Food supply is the most important of these. The number of organisms present in a given water depends upon the amount of organic matter (decomposing plant and animal matter) present in it, serving as food; the greater the amount the larger the number of microbes. Temperature is another influencing factor. At low temperatures bacteria do not multiply and so their number is held in check. A warm temperature favours rapid multiplication of bacteria provided there is sufficient organic nutriment in the water. Pathogenic bacteria, like the typhoid bacillus, are unable to survive long in the presence of rapidly multiplying saprophytes but are quickly destroyed. Special waters will have special types of organisms; for instance, in hot springs and sulphur springs thermophilic and sulphur bacteria respectively will naturally predominate. Bacteria in the surface layers of water are also being constantly destroyed by the sun's rays (actinic rays), thereby tending to keep down the number. This sterilising action of sunlight is more marked in the tropics where it is often utilised as a preliminary process in the purification of water by slow filtration. Two other factors that undoubtedly play a role in purifying natural waters, at least some types, are the acidity and the saline content; both tend to limit the number. The influence of rainfall and natural filtration has been previously referred to. Again, in some waters at least, bacteriophage exerts a lethal and consequently purifying effect. An example of this is the remarkably destructive effect of the Ganges water on *V. cholerae*.

The bacterial content of ice depends largely on the numbers present in the water from which the ice is formed. The majority of bacteria are killed when water is frozen. The rest of them remain viable for varying periods, but they do not seem to multiply. This is also true of some of the pathogenic bacteria. Less

than one per cent. of the typhoid bacilli has been found to survive after a week's freezing. Ice, therefore, cannot be considered entirely safe. Six months' preserving seems to make it sterile. Ice and ice products, like ice cream, may serve, though rarely, as transmitting agents of typhoid infection.

Bacteriological Analysis of Water. From what has been said above it will be clear that water may contain pathogenic organisms in addition to various saprophytic microbes both native to it and derived from extraneous sources. It is, therefore, unsafe to use any of the natural waters, particularly the surface waters, for drinking purposes without investigating the nature of its bacterial flora and without purification, if found necessary. The relative fewness of the pathogenic germs in the excreta, their great dilution in water and the predominance of the non-pathogenic flora all make the direct search for the pathogens extremely difficult. Usually it is not attempted in the routine bacteriological analysis of water. Sometimes it may become necessary to do it when special methods have to be employed. Excretal pollution of any water is accepted as contraindicating its use for drinking purposes and the presence of *Bact. coli*, *Cl. welchii* and *Str. faecalis* is regarded as indicative of such pollution; by themselves these organisms, it may be noted, are not harmful. Though all these organisms are always present when there is faecal pollution, the latter two are less numerous than *Bact. coli*. **Enterococci** and *Bact. coli*, though hardier than the typhoid bacillus, do not survive for long. Besides, the differentiation of the streptococci is often difficult to make. Also, the presence of spores of *Cl. welchii*, as they remain viable over long periods, does not indicate whether the contamination was a recent or a remote one. The demonstration of *Bact. coli* is also relatively simple. For these reasons *Bact. coli* is taken as the best indicator of faecal pollution. Hence, the immediate object of testing a water sample is to discover the presence, and probable concentration, of *Bact. coli* in it. A simultaneous examination for the detection of *Cl. welchii* and enterococci is also undertaken. In many laboratories the enumeration of the total bacterial population of water samples also forms part of the routine test.

The search for the presence of *Bact. coli* is based on the fact that this organism ferments lactose, producing both acid and gas. The usual procedure is to determine the presence or absence of

coliform bacilli in the sample and, if in the affirmative, to make a quantitative as well as qualitative estimation of them. MacConkey's broth is inoculated with 50 c.c., 10 c.c., 1 c.c. and 0·1 c.c. of the sample, inoculating a set of five tubes with each volume except the first. After incubation at 37° C. for two days the tubes are examined. Fermentation with the production of acid and gas indicates the presence of the coli group of organisms. The result is reported in one of two ways: coliform organisms present in 10 c.c., absent from 1 c.c., or in terms of the probable number of these organisms per 100 c.c. (presumptive coliform test), which is calculated from the number of tubes in which fermentation has occurred with the production of acid and gas. For this purpose a probability table has been worked out and a reference to it will give the presumptive coliform count. The coliform count offers an approximate measure of the extent of faecal pollution in the water. If the count is beyond a prescribed limit of safety, the next step is to make a qualitative analysis of these coliform types. For this purpose materials from 2 or 3 positive tubes containing the smallest quantities of water are inoculated on a suitable solid selective medium, such as MacConkey's agar. After incubation for a day, 2 or 3 acid colonies are picked from each plate and subcultured separately. These cultures are then studied with reference to motility, Gram's stain and biochemical reactions to differentiate between *Bact. coli*, intermediate and aerogenes-cloacae types (p. 424).

Coliform Bacilli. All the coliform bacilli that are found in water do not form a homogeneous group. Nor are they always inhabitants of the intestines. *Bact. coli* is a normal inhabitant of the animal or human intestines; it seldom leads a saprophytic existence and its presence in water is regarded as a sure proof of recent faecal pollution. The related *Bact. aerogenes*, another coliform organism that may be found in water samples, appears to be mainly distributed in grains, dust, soil and other situations. It has also frequently been isolated from the faeces. Others, such as the intermediate, *Bact. cloacæ* and the irregular forms, are also most probably non-excretal in origin. Hence, many authorities regard that the presence of the aerogenes-intermediate group is indicative of soil and not of sewage or excretal contamination and has, therefore, little or no sanitary significance. In waters they may be found alone without faecal coli. It may

indicate one of two things: either contamination has occurred with soil or faecal pollution has occurred in a sufficiently remote past, causing the disappearance of all faecal coli. Their great preponderance over *Bact. coli* in a water may also sometimes be more in favour of soil pollution, the coli being regarded as of animal origin, or a remote than of recent faecal contamination. These types are found more frequently and in relatively larger numbers in the tropics, presumably because of greater opportunities of pollution with dust and soil. It is maintained that no special importance need be attached to such findings, but the deciding factor in such cases should be the number of faecal coli. In such circumstances, therefore, much reliance should not be placed on the presumptive coliform count, but qualitative estimation should be proceeded with.

It may not be safe, however, to place too much emphasis upon the differentiation between *Bact. coli* and the aerogenes-intermediate group in routine water analysis. Coliform bacilli of any type are practically absent from virgin soils. On the other hand, *Bact. aerogenes* has been often isolated from faeces and urine. Also, this organism has not been universally absolved of all pathogenic role. It has been argued that *Bact. aerogenes*, being more hardy, survives over long periods in soil and water and that the less resistant *Bact. coli* dies out quicker than the former. But that alone is insufficient testimony to assume that when only the aerogenes and intermediate forms are present it negatives recent pollution. For the above reasons it may not be wise to regard their presence as altogether unimportant till we know more about their ecology and role in health.

Plate count forms part of the routine water analysis. An estimation of the total number of all types of organisms present per cubic centimeter of water is made. Though it is only a less reliable index of faecal pollution than the coliform count, it is of some value. A grossly abnormal number of organisms in any water, even though they may be saprophytes, renders it unwholesome for drinking purposes. Mention has been made before that many of the water and soil bacteria do not grow in the usual media used for water analysis and consequently the plate count does not give a correct estimation of all the bacterial population of water. Further, bacteria often remain in aggregates, either alone or clinging to decaying matter; such a group would give

rise to only one colony, leading to a totally erroneous count. Also, not all bacteria present may be viable.

The medium employed is a standard nutrient agar. Pour plates are made with different quantities of water, 0·01 c.c., 0·1 c.c., and 1·0 c.c., using 1·0 c.c. volume of decimal dilutions. Duplicate sets of plates are inoculated; one set is incubated at 20° C. for three days and the other at 37° C. for two days. Nutrient gelatin may be employed instead of agar for the set to be incubated at 20° C.; it is superior to agar for the growth of the saprophytic organisms but difficult to work with under tropical conditions. After incubation, the number of colonies in each plate is counted. The result is expressed as the average per cubic centimetre for the hot and cold plates separately.

In order to arrive at a correct interpretation of the results, it is necessary to take cognizance of several attendant circumstances. A correct sampling and great technical efficiency are both very essential. It is necessary to know the nature of the source, whether it is surface water or deep water and whether there has been any recent rain or flood. Detailed information about the source of water and its environments and a knowledge of the topography of the gathering ground are also very impor ant. The possibility of contamination with animal or bird excreta containing the coli group of organisms should be kept in view. Sources of water in the vicinity of pastures and heavily manured fields are likely to contain *Bact. coli* and if this is not taken into consideration, it may lead to a faulty inference and undeserved incrimination of the source. The same source, be it remembered, may vary in its bacteriological quality according to the seasons; this again depending chiefly on temperature and rainfall. Again, a single bacteriological examination, unless it reveals gross pollution, is of very limited value. Finally, in determining the potability of water, the results of bacteriological assay should be considered in conjunction with the results of chemical analysis.

The saprophytes grow best at 20° C.; in fact, many of them do not grow readily at 37° C., whereas the intestinal bacteria grow best at the latter temperature. Hence, the plate counts at the two temperatures may offer some suggestive evidence of the origin and type of the bacteria present. A high plate count at 20° C. alone may not have much significance. On the other hand, a high plate count at 37° C. is more valuable; it is often sufficient

o condemn a water because the greater the number of intestinal bacteria present in a water, the greater are the chances for the presence of pathogens in it. But usually plate counts are considered along with the results of coliform tests.

The coliform count is a more delicate test than the plate count at 37° C. While the presence of a large number of saprophytes is practically harmless, the presence of even a few coliform bacilli should be regarded as potentially dangerous. But the mere presence of this latter is not always sufficient to condemn a water supply; their number may be too small to be of serious import. If there are only one or two coliform bacilli per 100 c.c., the water is sufficiently pure for consumption. If the number exceeds ten, it indicates almost certain pollution and the water should not be recommended for drinking purposes without purification. If the number lies between these two limits, differential tests are indicated. If the coliform bacilli prove to be exclusively non-faecal in origin, the water may be passed fit for consumption, but a close watch should be kept on it. The relative significance of the members of the coliform group with reference to water sanitation has been discussed above.

Bacteriological Standards. No absolute bacteriological standards have been laid down for universal application; nor is it possible in view of the wide variations encountered in the bacterial flora of different waters. Every source should be considered on its own merits. The results of a single test, performed even in the best of circumstances, do not warrant a more than tentative opinion. By repeated examination of the same source, a fairly reliable standard can be fixed for it and any but slight departures from it should be viewed as a danger signal, demanding immediate attention.

Certain working standards have, however, been recommended. Those recommended by the English workers are summarised below:

	Deep waters	Surface waters
Plate count, agar, 3 days 22° C.	10–200 per c.c.	50–500 per c.c.
Plate count, agar, 2 days 37° C.	1–10 per c.c.	5–30 per c.c.
Bact. coli	less than 1 per 100 c.c.	less than 5 per 100 c.c.

	Deep waters	Surface waters
Faecal streptococci	less than 1 per 100 c.c.	less than 5 per 100 c.c.
Cl. welchii	less than 1 per 100 c.c.	less than 5 per 100 c.c.

A deep water that conforms to the standard given above is safe enough for drinking purposes. While a surface water that does not transgress the above limits may be permitted for human consumption, it requires a vigilant watch. For detailed discussion about the standards, reference may be made to "The Bacteriological Examination of Water Supplies" Ministry of Health Series No. 71, His Majesty's Stationery Office, London, 1934.

Milk

Milk is an important and common article of diet, particularly for children. Unlike water, it is an excellent medium for the growth of many pathogenic micro-organisms. Milk is subject to microbial contamination from several sources. The contaminating organisms may be pathogens, and milk may thus serve as a medium for the dissemination of infectious diseases. Hence, the great importance of the study of the bacteriology of milk has been recognised and legislative measures taken for the control of milk and milk products in many countries of the West. India has not yet begun to move in this direction.

Milk is usually obtained from cows, but in some countries buffaloes, goats, mares or reindeers are also employed as sources of milk.

In healthy cows the freshly secreted milk is sterile, but soon even in the udder of the cow it is invaded by bacteria getting through the milk ducts of the teats. Hence, even freshly drawn milk contains some bacteria, the fore-milk showing a far greater number than the strippings. During milking and subsequent storage, milk gets further accession of bacterial flora from various extraneous sources, such as the skin of the udder, the milkers, the milking vessels, and the dust from the barn and fodder. These organisms multiply rapidly and their number at any time depends upon the temperature of storage and time that has elapsed after milking. As there is no bacterial flora native for milk, the presence of micro-organisms is a certain evidence of contamination.

The various types of microbes, contaminating milk and their ources, are briefly dealt with below.

Infected Cattle. The milk may get its contamination from he tissues of the infected cattle. *Myco. tuberculosis* is the most mportant of this group. The bacilli get into milk directly from a tuberculous udder or they may be excreted into it from tuber- ulous lesions in other organs. Milk so infected causes the bovine ype of tuberculosis in man. In Britain and America it is a serious danger to the community, but fortunately it is relatively uncommon n India. This uncommonness may also be attributed, among ther factors, to the habitual boiling of milk before consumption. Another organism of this group is *Br. abortus* which is excreted n the milk of infected cows. *Br. melitensis* is similarly found n the infected goat's milk. Both these organisms cause undulent fever in man. The cow is often a victim to streptococcal mastitis when this organism is found in its milk. Epidemics of streptococcal sore throat are often associated with mastitis in cattle. Very uncommonly diphtheritic ulcers of the udder may occur and milk is liable to be contaminated with *C. diphtheriæ*. The virus of foot and mouth disease is present in the milk of cows suffering from this infection; the disease it causes in man is very mild.

External Sources. Milk also receives contamination from external sources. The contaminating organisms may be patho- genic or non-pathogenic. Obviously, the former is far more important from a hygienic view than the latter. Infection of this type occurs mainly from milkers or others handling milk who are carriers of infection or who happen to be actual cases but of a mild type. For example, the streptococcus or the diphtheria bacillus can be transmitted directly from the throat to the milk by coughing or sneezing or the enteric, dysentery or cholera bacilli through the hands of a carrier. Contamination can also enter indirectly through the water used for washing the milking utensils or rarely through infected cow dung. Milk is not an indifferent medium like water, and, in whatever way contamina- tion takes place, under favourable conditions the pathogenic bacteria multiply rapidly in it.

Contamination with non-pathogenic organisms of extraneous origin may take place in a variety of ways. The skin of the udder and teats may contain soil, manure and other materials. If before

milking the udder is not properly cleaned, these materials may gain access to milk, contributing largely to its bacterial flora. This is true also of the hands of the milkers, but the greatest source of contamination is the milking equipment including the utensils. Dust also contributes its quota, though the number of organisms from this source may not be great. If water has been used as an adulterant, the kind and quantity of contamination depend upon the water added. Members of this category do not cause disease in man, but by their activity they may bring about alterations in the physical state of milk, rendering it unsuitable for consumption.

Milk drawn under the best of conditions contains only relatively few organisms, 200–400 per cubic centimetre. But the initial number will be severalfold when conditions of production are not under adequate control. However introduced, the bacteria multiply rapidly in milk and their concentration at any time depends mainly upon three factors: the temperature at which milk is stored, the time that has elapsed after milking and the degree of the original infection. Preservation in the cold directly after milking keeps multiplication of bacteria under check. But if the milk is kept at higher temperatures, rapid multiplication of the contained bacteria ensues, giving rise to an enormous number per cubic centimetre. It is obvious, therefore, that, apart from the health of the animal, such factors as cleanliness in the collection and transport and the temperature of storage are also intimately concerned with the sanitary quality of milk.

The bacteria present in milk may be considered under two groups: the pathogenic and the non-pathogenic. The former is dealt with above. The latter may again be classified as follows:

Acid-Forming Bacteria. The acid-forming bacteria are those organisms which are concerned with the natural fermentation of milk. The commonest type of spontaneous fermentation is the lactic acid fermentation, occurring during souring of milk under natural conditions. The milk sugar, lactose, is fermented with the production of acids, predominantly lactic acid, which, acting on the soluble caseinogen, convert it into the insoluble casein; this is precipitated in the form of soft flaky or lumpy mass commonly known as curd. The organisms which are usually active in this process belong to the *Streptococcus lactis* type. Other common species like *Staphylococcus aureus* and *Streptococcus pyogenes* may also bring about natural fermentation of milk.

Lactic acid fermentation can also be induced by inoculating milk with members of the *Lactobacillus* group, such as *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. These organisms normally occur in small numbers in the human mouth and intestines. They seem to have no part in the natural fermentation of milk.

Alcoholic fermentation of milk under natural conditions occurs only rarely. But in some parts of the world it is induced for the preparation of certain special beverages from milk. This, for instance, is the process of preparing koumiss, a drink prepared by Tartars from mare's milk; the action is brought about by the agency of yeast and lactic acid bacteria. Koumiss contains both alcohol and lactic acid. Certain species of yeast can directly ferment lactic acid and produce alcohol.

Gas-Forming Bacteria. They ferment lactose with the production of both acid and gas. Acetic acid is the chief acid produced, which gives an unpleasant flavour to the curd. Gas production is abundant and the curd is disintegrated. Coliform bacilli, including *Bact. aerogenes* probably introduced from cow dung or fodder, and certain anaerobic organisms, such as *Cl. welchii* and *Cl. butyricum*, are the commonest organisms concerned with this type of fermentation.

Alkali-Forming Bacteria. These organisms do not attack lactose. Apparently their action is on the protein content of milk, leading to the formation of ammonia. Important members of this group are *Bact. alkaligenes* and certain spore-bearing bacilli. When non-lactose-fermenting organisms, like the typhoid bacillus, grow in milk, a slow increase of pH is the result; no other change is observed. It is not uncommon to see milk converted into a yellowish transparent fluid. This is due to the activity of certain aerobic spore-formers which, by elaborating a lipase during growth, saponify the fats present in milk.

Proteolytic Bacteria. These organisms, besides increasing the pH, bring about hydrolysis of the milk proteins. This they do by the agency of two enzymic systems: rennet and casease. The former acts in two stages: first there is the conversion of caseinogen into a soluble form of casein. The casein then combines with the calcium salts in solution and is precipitated as insoluble calcium caseinate. The curd so formed is surrounded by a clear fluid called whey; the whey contains the lactose, salts and other proteins (lactalbumin and lactoglobulin) in the milk.

Casease is a proteolytic enzyme which digests the proteins, resulting in the final conversion of the milk into a clear fluid, a process often referred to as *peptonisation*. Both the enzymic systems may not be present in the same organism, when only the action corresponding to the one present takes place. Organisms responsible for such changes are certain aerobic spore-formers, like *B. subtilis*, *B. mesentericus*, *Proteus vulgaris* and certain strains of streptococci.

Occasionally, certain chromogenic bacteria get access to milk and cause abnormal changes with the formation of coloured products. Thus are produced "blue milk" by *Pseudomonas cyanogena*, "red milk" by *Chromobacterium prodigiosum* and "yellow milk" by *Bact. synxanthus*.

Inert Bacteria. These do not produce any visible changes in milk. They may include both pathogenic and non-pathogenic bacteria. Only by bacteriological examination can their presence in milk be revealed.

The Bactericidal Property of Fresh Milk. Freshly drawn milk is definitely bactericidal and bacteriostatic. This is, no doubt, responsible for the initial fall in the bacterial count of fresh milk. This property disappears in a few hours after the milk is drawn; it is also thermolabile, being destroyed in fifteen minutes at 75° C.

The Determination of the Quality of Milk. Several methods are in use for testing the sanitary quality of milk. The bacterial count is probably the most reliable index. Water has its natural bacterial flora, but milk has none. Hence, the presence of organisms in milk is a sure indication of contamination, and their number is a direct, though approximate, measure of the degree of contamination.

Plate Count. The plate count gives only an approximate idea of the number of organisms present in milk. Nevertheless, it is in general use for the grading of milk. Proper sampling and the employment of standard media and standard technique are essential. The procedure is similar to that employed for water, *viz.* dilution and plating and counting the number of colonies after a definite period of incubation. The criticism that plate count is not an accurate reflection of all the bacterial population in a water sample is equally true of the plate count in milk analysis. The bacterial count depends upon several factors and

varies under different conditions and in different localities. Consequently, no rigid limit applicable in all circumstances can be set. In England "Tuberculin-tested (pasteurised)" milk must not contain more than 30,000 organisms per millilitre and must not contain *Bact. coli* in 1/100 ml. and "pasteurised" milk must not contain more than 100,000 organisms per millilitre. In some parts of America the bacterial count of Certified milk is limited to 10,000 organisms per cubic centimetre. In the same locality it is not desirable to have several standards as that would favour a greater marketing of inferior quality milk.

Breed's Microscopic Count. In this method 0·01 c.c. of milk is discharged on a slide by means of a capillary pipette and spread over an area of one square centimetre on the slide. After drying, the fat is dissolved out with xylol; the smear is then fixed with alcohol and stained with methylene blue. The number of organisms in a measured area is counted and from this the number per square centimetre is calculated and finally the number of organisms per cubic centimetre of the original milk is estimated. It is a rapid method involving no incubation and is of special value in collecting stations where within a few minutes the quality of the milk from the individual farms can be judged and those that are unsatisfactory returned to the producer. Thus a mixing of good and bad quality milk can be prevented.

Methylene Blue Reduction. This is a simple test based on the fact that the growing organisms form certain metabolites capable of reducing methylene blue into a colourless leuco base. If milk is mixed with a small amount of methylene blue (10 c.c. of milk *plus* 1 c.c. of standard methylene blue solution) and incubated, the colour is discharged in course of time. The incubation should be conducted in darkness and the tubes should be inverted every half an hour in order to ensure uniform mixing. Though freshly drawn milk itself exhibits the reducing property, it is negligible and the action may be considered as practically due to the bacterial activity. The reduction time is, therefore, roughly proportional to the bacterial content of milk. Though not very precise, it is, nevertheless, a useful test for the control of raw mixed milk. A milk decolourising in less than two hours is definitely poor in quality, while one which takes more than eight hours is of very good quality. It is claimed that this test is a better index of bacterial growth than the plate count.

The Coliform Test. The Coliform test is a reliable index of the excretal pollution of water, which includes the probability of pollution with human pathogens. But in the case of milk it has not the same significance. A very large proportion of mixed milk contains coliform organisms. Contamination in these cases is most likely through cow dung, fodder and dust or from imperfectly cleaned vessels. Contamination from these sources is not so objectionable as when it is with human excreta. Coliform bacilli do not multiply in water but tend to die out, whereas they multiply rapidly in milk. Thus, a few organisms introduced multiply soon into a large number. For this reason the presence of coliform bacilli in milk in larger numbers than is permissible in water may be tolerated, and their differential studies are not now considered necessary except under special conditions.

The Isolation of Pathogenic Bacteria. The isolation of pathogenic bacteria from milk is, in contrast to that from water, more simple and practicable. For example, the presence of tubercle bacilli in milk can be conclusively shown by guinea-pig inoculation. After thorough mixing two samples of 50 c.c. each are centrifugalised. The sediment from each is suspended separately in 2·5 c.c. of sterile saline solution and injected into the groin of a guinea-pig, thus employing two animals for the same milk. The animals are kept under observation. One is killed at the end of four weeks and an autopsy is made; if a thorough search reveals no evidence of any tuberculous infection the second animal is allowed to go on for eight weeks, when it is killed and examined. Though reliable, the test is costly and takes several weeks for completion. The tubercle bacilli may also be demonstrated by cultural and microscopical methods. For culture the deposit from milk is treated appropriately with either NaOH or H_2SO_4, neutralised and inseminated on a selective medium. The results are read in two to three weeks. In the microscopical method, smears from deposits are stained by the Ziehl-Neelsen technique and are examined under the low power for the presence of cell groups. These are collections of endothelial cells, and tubercle bacilli, when present, are often seen in and among these cells. These tests are, of course, not so reliable as the pathogenicity test. The cultural method for the detection of other pathogenic bacteria, like streptococci or the *Brucella* organisms, are rela-

tively easy. Samples of fore-milk from individual quarters should be separately tested for this purpose.

The Sediment Test. This is a test for the cleanliness of milk and not so much for its safety. The amount of extraneous dirt in milk can be determined by this method. The test is conducted either by filtration through a standard cotton disc or by centrifugation. It is a simple and useful test in controlling gross uncleanliness in milking. But it is to be remembered that a producer by a previous straining through muslin can defeat this end.

The Cellular Count. Various types of cells are found in normal milk; their number also varies. An abnormal number of leucocytes, as noted by the Breed's method, suggests mastitis. The presence of streptococci in large numbers may be an additional proof. But retention of milk in the udder, by causing desquamation of cells, may also lead to a high cell count. Hence, mere cell count is only of limited value.

The Hygienic Control of Milk. In no part of India is there any legislation for the control of milk supplies. But in many Western countries organisations for providing general protected milk supplies have been in existence for long. In these places regulations have been laid down for the control of milk at every stage during production, collection and distribution to the consumer. Where such regulations are strictly enforced, milk supply has reached a high degree of safety with the result that milk-borne diseases have been largely controlled. Even in such countries it has not been possible to lay down one uniform standard. Consequently milk with different grades of hygienic quality such as Certified milk, Grade A milk, Tuberculin-tested milk and so on, are available in the market. But there is a growing tendency to confine to one rigid standard, as otherwise, the marketing of milk of lower standard would become too common to be safe for the community.

The hygienic quality of a milk supply can be controlled in two ways. One is by preventing, as far as possible, the contamination of milk and the subsequent multiplication of the contaminating bacteria and the second by destroying the contained bacteria, particularly the delicate pathogens, by suitable methods. Both these measures should be applied wherever possible so that the safety of milk can be doubly assured.

Reference has been made to the several probable sources of contamination of milk. The infected animal is an important source, especially of the pathogenic bacteria. Hence, the control of animal diseases and the elimination of sick animals from milking are the first steps. But these are not simple matters and the co-operation of many interests is necessary to put them into practice. They are also very costly. A no less difficult, but equally dangerous, source to control is the human carrier and people suffering from mild infections, who may be among the personnel handling milk. The ideal is the non-employment of all such persons in the milk industry. Examination of all the milk operatives and this at sufficiently frequent intervals to be of real value requires an elaborate and costly system. Other factors concerned with the collection and distribution of milk, such as the cleanliness of barn-yard and dairy, the purity of the water supply of the farm and the cleanliness of milking equipments, can be more easily controlled. For this purpose regular inspection of dairy farms and strict enforcement of regulations are quite essential.

The destruction of pathogenic bacteria in milk is the surest way of ensuring a safe milk supply. This is best done by the application of some form of heat. Boiling, pasteurisation and sterilisation are all methods in which different degrees of temperature are employed. Individual milk can be easily rendered safe by boiling. In this country boiling is probably the only method employed; but it is done almost universally and unboiled milk is seldom consumed. Boiling and sterilisation destroy all forms except the most resistant spore-bearing bacteria. But such processes have the disadvantage of causing radical alteration in the quality of milk. From this point of view pasteurisation, first applied by Pasteur in the preservation of wines, is the most satisfactory.

Pasteurisation. In this process the temperature is raised just enough to kill most microbes but not too high to produce any significant changes in the physical state of milk. This process is applied with slight modifications in different places. A satisfactory method is to raise the milk to a temperature of 145°-150° F., hold it at that temperature for thirty minutes and to cool it immediately to a temperature of 55° F. or less. A criticism levelled against pasteurisation is that it causes a partial

destruction of vitamin C and possibly of iodine. But this is insignificant compared to the grave danger of infection and in any case such losses can be easily made good by other means.

Milk Products. Various milk products, such as ice cream, butter, cheese and others, may contain pathogenic bacteria and act as vehicles of infection, if the milk from which they are manufactured is obtained from questionable sources. It is true that such organisms tend to die out on keeping. But certain pathogens survive over long periods. For instance, the tubercle bacillus may remain viable in butter and cheese fairly long. The role of ice cream in the transmission of intestinal infections, especially typhoid fever, has already been referred to. Food poisoning and *Brucella* infections are others that may be caused by the agency of milk products.

CHAPTER X

INFECTION

Bacterial infection is a frequent cause of disease, far more frequent than any other cause. A large number of definite diseases, many of which may cause wide-spread and violent epidemics, are caused by the pathogenic micro-organisms. In addition, microbes are responsible for a great many disabilities and minor ailments which, by undermining the general or focal resistance of the host, may prepare the ground for other serious infections or non-bacterial diseases. Naturally, therefore, all the circumstances concerning infection are of supreme importance to us.

As infection involves two living entities, the attacking parasite and the host, it cannot be a simple unilateral action of the invading parasite. On the other hand, infection is followed by a vigorous reaction on the part of the body which attempts to mobilise all its resources to combat infection and dislodge or destroy the invading agent. Obviously then, infection consists of a complex process of action and reaction between two contending forces, those relating to the attacking parasite and those marshalled by the invaded victim. The outcome of this interaction expresses itself as infectious disease. It is self-evident, therefore, that a thorough knowledge of the intimate nature of these reacting forces is essential for a proper understanding of the clinical sequel of infection. An insight into the manner of the individual's natural reaction to infection teaches us how to imitate nature and evolve valuable means of protecting the individual against infection. Its practical application, again, places in the hands of the clinician very valuable methods of diagnosis, prognosis and therapy. From another point of view also the various factors leading to infection are of tremendous importance. It should be remembered that, besides the parasite and the host, the incidence and spread of infection are considerably influenced by the environmental factors. In mass infection

all these entities assume particular importance. A knowledge of their working and interrelationship may, and often does, enable us to strike at the weak links in the chain of mass infection and thereby control the spread of disease.

Infection is the successful invasion of the host tissues by the pathogenic micro-organisms with or without the production of symptoms. The mere presence of such bacteria in the body does not constitute infection. Infection implies growth and multiplication of the organisms in the tissue with production of poisonous products which cause tissue reaction and damage. Infection associated with the production of symptoms is disease. Sometimes infection is present without giving rise to clinical manifestations. The condition is then known as *latent infection* or *subclinical infection* (subinfection); the reaction here is at a subclinical level and so the presence of infection is inapparent. It is not always possible to draw any rigid distinction between this and the healthy carrier who imbibes infection and harbours it for varying periods after exposure and without developing any ill-effects from it. Infection may also remain quiescent and concealed in *chronic carriers* who even after recovery may harbour infection for long periods.

Distribution of Bacteria in Nature. Bacteria are present everywhere in nature, including the body surfaces of animals and man. Many of them live on dead organic matter and are called *saprophytes* (Gr. sapros, rotten, phyton, a plant). They fulfil an important function in the economy of life, *viz.* the reduction of complex dead organic matter into simpler substances readily utilisable by plants. Moreover, they are of great economic importance, playing an extremely useful role in industry, agriculture and other human activities. Others are *parasitic*, living upon other plants or animals. The division into saprophytes and parasites is by no means rigid or exclusive as some species exhibit both properties. The pathogenic bacteria are mostly of the parasitic type, living at the expense of some host, animal or man. Parasitism is a widely prevalent biological phenomenon found in nature and infection is obviously a special type of it.

The skin and mucous surfaces of the body are normally inhabited by numerous species of micro-organisms. Various regions, the intestines, the genitalia, mouth, nose, nasopharynx, conjunctiva and skin, have their more or less fixed normal

bacterial flora, for example Doderlein's bacillus of the vagina, *Staph. albus* of the skin, viridans streptococcus of the pharynx, xerosis bacillus of the conjunctiva, the colon bacillus and various types of spirochaetes and anaerobic and aerobic organisms of the gut (p. 163). They lead a commensal life and do no harm to the host. The term *commensalism* means a living together of two species in which one is benefited by the association, while the other is neither benefited nor injured. Some of the normal surface denizens are potential pathogens, *i.e.*, they are capable of taking on a pathogenic role under certain conditions. So long as they are in their natural habitat no harm is done to the host, but in certain circumstances they leave the normal surroundings, invade the tissues and cause disease. For example, *Bact. coli* inhabits the lower alimentary tract as a harmless commensal. When it gets an opportunity to leave the intestines and reach the tissues, it sets up inflammation, causing diseases such as cholecystitis, peritonitis, cystitis and so on. Another phenomenon that is observed in nature is the living together of different species of organisms, deriving mutual benefit. This is called *symbiosis*. There are numerous examples in nature of such intimate association with reciprocal advantage. Certain soil bacteria growing as nodules on the roots of leguminous plants absorb nitrogen from the air and transform it into some readily utilisable compound for the use of the plant, while, in turn, the bacteria themselves feed on the plant sap. Another instance is that of the cellulose-breaking bacteria living in the gut of wood-eating insects, such as the white-ants. No case of such symbiotic association is known in the case of man. Certain species of organisms are unable to live together, as some metabolic products and toxins generated by one, though harmless to itself, are, nevertheless, detrimental to the growth of the other. The antagonist may also act by depriving the other of the required pabulum for growth. The term *antibiosis* or *antagonism* is applied to this condition. For instance, *Lactobacillus bulgaricus* forms large amounts of lactic acid during the souring of milk. This high acidity is inimical to the growth of putrefactive bacteria in milk. The inhibitory effect exerted by *Ps. pyocyanea* on the growth of the diphtheria bacillus is another instance. Another well known example is the mould *Penicillium*, some species of which during growth produce metabolites (notably penicillin) which inhibit the

growth of many bacteria. Such growth-inhibiting factors naturally produced by micro-organisms are referred to as *antibiotics*. *Synergism* is a condition in which two or more bacterial species growing together produce a result which a single species alone could not have produced. For example, Morgan's bacillus and the typhoid bacillus growing together in mannite or maltose form appreciable amounts of gas which neither organism growing alone could have done. Vincent's angina is another illustration of the associate activities of bacteria, a spirochaete and a bacterium.

The vast majority of parasitic bacteria are incapable of invading and injuring tissues. It is a striking fact that only a few possess this property; they are called *pathogenic* which means disease-causing. The number of species capable of causing disease in man and animals is in the neighbourhood of hundred. From the point of view of medicine micro-organisms can then be conveniently classified into the pathogenic and non-pathogenic groups. It should be borne in mind that these are only relative terms; a rigid grouping into two such categories is impossible since an organism that is pathogenic to one animal species may not be pathogenic to another. For example, the gonococcus is pathogenic for man while inactive towards lower animals; *per contra*, the virus of rinderpest or cattle plague does not attack man. By the term pathogenic is meant that the organism in question can cause disease in some one species or other. During their association with man, different groups of pathogenic bacteria are found to have attained different degrees of parasitism. The same is also the case with pathogenicity. The hardy staphylococcus, growing luxuriantly on ordinary culture media, exhibits much less parasitic dependence than the delicate gonococcus; nor does the staphylococcus display the same degree of virulence as the tubercle bacillus or the plague bacillus.

Most of the pathogens are not normally present on the body but reach it from outside. After establishing a foothold in the body, they multiply and form injurious products which damage tissues and stimulate reaction. This process of successful invasion and injury constitutes infection.

Specific Infection. The struggle put up by the host against an invading bacterium, as evidenced by clinical manifestations, constitutes infectious disease. When a particular organism gives rise to the same type of reaction and symptomatology in the same

host species, the disease is referred to as specific infectious disease, *e.g.* diphtheria, tetanus, cholera and others; when the symptoms are acute, acute specific infectious diseases. Some acute ones may become chronic and some chronic infections may take an acute turn after varying periods of chronicity. Sometimes the same symptom complex is caused by different organisms, for example meningitis, bacillary dysentery, bronchopneumonia, endocarditis. Again, in certain lesions such as gas gangrene and boils more than one species of organism are present. Such infections are not specific. A specific infection is in all circumstances caused by the same organism. How to establish this causal relationship between a given organism and a particular disease has been summarised in what are commonly known as *Koch's postulates.* They are:

1. The organism should be found in all cases of the disease in question, and its distribution in the body should be in accordance with the lesions observed.
2. The organism should be cultivated outside the body of the host, in pure culture, for several generations.
3. The organism so isolated should reproduce the disease in other susceptible animals.
4. The organism should always be found in, and recoverable from, the lesions of the reproduced disease.

It is doubted whether Koch ever enunciated these postulates in such categorical terms. At any rate, the rigid application of these conditions is neither practical in every infection nor necessary. By ordinary methods it is not possible to demonstrate the presence of viruses in the tissues in diseases of accepted viral aetiology. Their presence can be shown only by animal experiments. Viruses have not been grown in cell-free media; some of them may be grown in tissue culture or in the developing chick embryo, but it cannot be asserted that such growths are pure, and not mixed cultures. Similarly, the leprosy bacillus has not been cultivated, but still its causal relationship with leprosy has been universally accepted; the same is true with regard to *Sp. minus* of rat-bite fever. Again, in the case of many pathogenic bacteria no susceptible animals have been discovered, as instanced by the gonococcus, meningococcus and certain viruses. Where susceptible animals are available, the disease reproduced

in them by the injection of the isolated organism does not always correspond clinically to the original disease in man. For example, there is little clinical identity between the condition produced in experimental animals by the typhoid germ and the typhoid fever in man. Curiously enough, phylogenetically related species, like monkeys, the anthropoid apes and others, are in general no better experimental animals than the distant rabbits, guinea-pigs and the like. The fourth postulate is in a way a corollary of the first three and when an organism complies with these, it will generally be found to satisfy the last also. From the above comment it is clear that the application of any or all these postulates in the case of all infections is not easy or possible. However, a great many infections can be shown to fulfil them. In any case these postulates should be applied as the confirmatory criteria where doubt exists.

In practice, the fulfilment of these postulates is not always necessary for incriminating a particular bacterium as the cause of a disease, nor is it sought for in most of the routine diagnostic procedures. The weight of ancillary evidence may often be sufficient to prove the causal relationship of a suspected organism to a disease. Certain specific reactive products, cellular or humoral, have been shown to afford satisfactory basis for developing confirmatory diagnostic tests. For example, the finding of typical *giant cell systems* in a suspected case of tuberculosis or of *Negri bodies* in rabies is accepted as diagnostic for all practical purposes. But it also appears more than likely that the Negri bodies and similar inclusion bodies may ultimately be proved to be viral aggregates, when a diagnostic test of a more or less empirical nature will be lifted to the status of a scientific proof. The demonstration of specific antibodies in the blood of the infected host by any of the immunological tests, such as the agglutination, complement-fixation or allergic test, furnishes sufficient evidence for fixing the aetiological responsibility of an organism in a disease even if the organism cannot be found in the lesion or cultivated therefrom. However, be it emphasised again that these are only ancillary aids and the final proof, when necessary, should be sought for by the application of the postulates.

Source of Infection. It is very important to know where exactly in nature do the pathogenic bacteria reside. This knowledge often enables to get at the very root of infection and is an

essential prerequisite in devising means for the control of spread of disease. In all cases the ultimate source of infection is *man*, *animals* or *soil*. Of these, the first two are far more important than the last. For, as mentioned above, most of the pathogenic microbes are adapted to a parasitic mode of existence and cannot live in the external world save for a relatively brief interval of time. Water serves only for a temporary sojourn and not as a permanent abode; so also air. The individual himself may act as the source of infection or infection may be communicated to him from another man. Mention has been made above that some of the pathogenic bacteria live on man either as commensals or in a quiescent state and in the event of temporary depression of tissue resistance, they are liable to initiate disease. This type of auto-infection is referred to as *endogenous*, as opposed to the *exogenous* type in which the infecting agent invades the body from without. Man serves as an external source of infection mostly as the result of pre-existing disease, which may be acute, subacute or chronic, as in the case of cholera, enteric, influenza, smallpox, gonorrhoea, syphilis, tuberculosis and others. Equally, or in some respects more, important is *atypical infection* which passes off with manifestations not characteristic of the particular disease. Again, the human reservoir may be a latent case or a carrier, as for instance the enteric, meningococcal or dysentery carrier.

The reservoirs of infection in certain diseases are the lower animals and from close association with them or through food relationship the infecting agent is communicated to man. Illustrative of such exogenous infection are tuberculosis, plague, rabies, undulent fever, anthrax, glanders, rat-bite fever and actinomycosis. A few of the pathogenic bacteria live normally in the soil and man gets the infection through contaminated wounds. The tetanus bacillus and the gas gangrene organisms are examples of this.

Method of Transport. Infection may be transmitted to man directly from the source or indirectly through intermediate agencies. In the case of some diseases, like the venereal diseases, yaws, coccal infection, operation or post-mortem infections, the infecting agent from the sick gains entry into the healthy man through direct contact. These are contact infections and in them intermediate agents play no role in transmission. Contact is essential for spread in some of these diseases, *e.g.* syphilis

gonorrhoea and yaws, as their aetiological agents are quickly destroyed outside the body. The term *contagious disease* used to be reserved for diseases communicated through contact. Now no such rigid application is enforced and the term is frequently employed to denote all bacterial diseases which are directly transmissible. Some of the animal diseases are also transferred to man through direct contact, *e.g.* rabies. The pathogenic bacteria normally found in the soil, such as the tetanus and gas gangrene organisms, are likewise transmitted to man directly from the source. The infected material (soil) in these cases is implanted directly into the tissues as a result of injury.

Another very common method of direct spread is through droplets and sprays discharged during speaking, laughing, coughing and sneezing—*droplet infection*. It is believed that the infected droplets may be directly inhaled by the neighbouring healthy persons who thereby acquire infection. This may not be the whole story. The mechanism of air-borne infection has been elucidated by recent experimental studies. Soon after expulsion by sneezing, laughing or coughing and before traversing forward sufficiently long to cause infection, infective droplets greater than 0·1 mm. in diameter drop to the ground and so do not constitute efficient transmitters of infection through air. Particles smaller than this are evaporated before reaching the ground, but they leave suspended nuclei consisting of organic matter, salts and bacteria. How long these organisms can remain viable and virulent in the atmosphere depends largely on their resistance to drying. Evidence so far as it goes tends to show that some of the respiratory pathogens may in these circumstances maintain their vitality and virulence for several hours. The significance of crowded rooms and rooms occupied by patients in the transmission of infection is, therefore, obvious. Many important epidemic diseases are spread by the droplet method; the common cold, the pneumonias, tuberculosis, diphtheria, scarlet fever, meningitis, influenza, smallpox, measles, mumps, chickenpox, and poliomyelitis are examples of this type. A characteristic feature of this type of infection is its rapid dissemination, particularly in a crowded community.

In indirect spread an external agency is necessary to carry infection to the healthy man. This may be *air* laden with infected dust, minute particles of dried sputum or cast off skin; *water*;

food and *fomites* such as eating utensils, wearing apparel and bedding. Intestinal infections necessarily take place through infected food and drink. When the transmission of infection is through contaminated water, as for example cholera and enteric fever, it is often referred to as water-borne infection. In this mode of transmission there is necessarily a preliminary contamination of the drinking water supply. Milk also may form the vehicle of infection in certain diseases common to man and animals, as exemplified by tuberculosis and brucellosis. The habit of sucking pencils, penholders and similar things by school children is a common mode of dissemination of disease in schools.

Insects may act as the transmitters of infection; those that do this are called *insect vectors*. They may be *portal* or *mechanical carriers*. Such vectors do not themselves suffer from the disease, nor carry the disease agent for anything but a brief period after receiving it. Very soon after getting the infected material in or on them, they transfer it directly to a new host or indirectly by contaminating food or drink. The contamination of food or drink with infected excreta by the common domestic fly is a well known instance of this sort of transmission. In the case of the other insect-borne diseases, the organism remains in the insect host for a definite period of time before it becomes transferable to a new host. Such carriers are called *intermediate hosts*. What happens to the organism during this interval is by no means clear. It may be a multiplicative phase meant for a concentration of dose or some form of rejuvenating phase, indispensable for the preservation of species. The important point is that during this latent period the insect is not infective to a new host. Other examples of insect vectors are the mosquitoes in yellow fever and dengue, lice and ticks in relapsing fever, fleas, ticks and mites in typhus and fleas in plague. These insect vectors are often ectoparasites of animals. The animal host, from which the disease agents are thus communicated to others, are referred to as *reservoir host*, *e.g.* man and many rodents.

Transmission of infection may occur mechanically by the agency of man. For example, a nurse attending on a case of puerperal sepsis may through inadequate attention to asepsis convey the infection to a healthy host; or a cook who is an enteric or dysentery carrier may contaminate food through insanitary habits and lack of personal cleanliness.

Human Carriers. Carriers are apparently healthy people who harbour infection. Though in these cases the infectious agents are apparently harmless to the host, they keep up their virulence and are able to cause disease in others. The carrier state may arise from the beginning as a silent symptomless infection or as a sequel to disease. In the former the condition develops insidiously following exposure to infection which may be a sick individual or another carrier. This type of carrier is known as *contact carrier* or *healthy carrier*. Common examples are diphtheria, meningococcal and poliomyelitis carriers. Contact carriers may be transient or temporary or may rarely become chronic. Sometimes there may be a slight reaction in them with the result that a low grade immunity may be developed.

Latent infection is also a carrier state and may play a part in the dissemination of infection. Between this and the contact carrier the difference may not be always appreciable. Both carry infection without giving rise to clinical evidence and both are able to propagate the infection. In both, the parasite may not be altogether inactive.

During recovery from a disease the body usually gets rid of the infectious agent quickly. Sometimes during convalescence the organism persists in the body for varying periods, but it is incapable of doing any harm as the body is protected from further injury on account of the immunity already developed in the course of recovery. This type of carrier is called a *convalescent carrier*. The organism persists in certain foci of infection where there may be frequently minimal reactions. The position of these foci may be such that they are not easily accessible to the antibodies and the organism, in its turn, is thus protected from their action. That is probably why body is not able to get rid of the infection readily. Most of the convalescent carriers soon become free from infection, but a few, a very small percentage, do not get rid of the infection for a long time, sometimes throughout life, and become *permanent* or *chronic carriers*.

The excretion of the infectious agent from the carrier is not continuous but intermittent, adding to the difficulty in their detection. The path of excretion depends upon the focus of infection; for example, a diphtheria carrier gives out the organism in the nasal or oral secretions, while an enteric carrier excretes it in the faeces. The organisms causing some of the intestinal

infections, such as dysentery and cholera, remain confined to the intestines; others like the enteric bacilli which invade the blood stream may set up foci of infection in various organs. In the former type of infection, therefore, the causative organism is excreted only in the faeces, whether it be an active case or a carrier. In the latter, on the other hand, there are two outlets for excretion possible, through faeces as well as through urine. Carriers that excrete bacilli in the faeces are referred to as the *faecal type* and those that excrete in the urine as the *urinary type*.

Carriers form an important source of infection; often far more important than actual cases. Because of the insidiousness, they are able to disseminate infection more widely and are, therefore, more dangerous to the society than actual cases. Carriers also constitute a difficult problem in public health work. No sufficient evidence is forthcoming to show that the organism excreted by a carrier is of attenuated virulence, though it is believed in some quarters that the bacteria in the carrier state may undergo variation into the rough avirulent form under the influence of the host's immunity. Equally insidious sources of infection, as mentioned above, are the atypical and the mild cases which go undiagnosed.

Portal of Entry. The possible gateways through which infection can enter the body are the large skin surface, the respiratory tract, the alimentary tract, the genito-urinary tract and the conjunctivae. Another is the transplacental route through which the developing foetus may get infection from the maternal blood. Very rarely infection may get in through an infected middle ear. The mechanism of transmission indicates the particular channel through which an invader finds its way into the tissues. When dust or droplet acts as the vehicle, the route of infection is necessarily the respiratory tract, the mouth or both. Similarly, when water, milk or food serves as the medium, the alimentary tract forms the path of infection. Contact infections are contracted by the direct deposit of the infecting agent on the skin or mucosa. Blood sucking insect carriers implant the infection through the skin into the tissues or blood. Thus, the particular channel of entry depends upon the method of transport of the invading microbe.

A pathogenic organism entering through any route will not always cause disease. The portal through which it enters the

body is an important factor in determining infection. Each microbe seems to be adapted to a particular route and for successful infection it should enter through that route. Otherwise, infection may not occur or, even if it occurs, will only be insignificant in nature. For example, a dose of the cholera or dysentery bacillus injected under the skin may cause at most a local lesion and not clinical cholera or dysentery. For any of these to happen the organisms should enter through the alimentary route. Similarly, if the virus of smallpox or the meningococcus is deposited on the skin nothing would ensue, but if either is inhaled into the respiratory tract, infection may follow. In the same way, the tetanus or the welchii bacillus would pass through the alimentary tract harmlessly, while their implantation into a wound would lead to infection and disease.

Another point to remember is that most of the pathogenic microbes are unable to penetrate through the intact healthy skin or mucous membrane and cause infection. There is, for instance, the white staphylococcus always residing on the skin and mucous surfaces without being able to invade the tissue so long as these structures maintain their vitality and health. But when there is an abrasion or trauma, the organism readily gains entry and sets up inflammation.

Prevalence of Disease. The individual's reaction to infection constitutes disease. The study of disease as confronted in the individual is clinical medicine. When a disease occurs suddenly in a community and spreads rapidly attacking many people simultaneously, it is spoken of as an *epidemic* or *mass infection*. The course of epidemic infection is really the natural history of disease in relation to the mass. Mass infection among animals is called *epizootic*. Examples of epidemic diseases are cholera, plague, pneumonia and smallpox. The conditions which govern the occurrence, evolution and control of epidemics form the subject matter of epidemiology. Of course, it also takes cognizance of disease in the endemic state. When a disease is constantly present in a locality it is said to be *endemic*, for example, plague in Bombay, Secunderabad, Hosur and other places where it exists as a smouldering infection all through the year. An endemic infection may occasionally break out in an epidemic form with explosive suddenness involving several people at the same time. A disease is said to be *sporadic* when few cases,

scattered about in point of place and time, occur in a locality. When a disease spreads in the form of an epidemic in several countries of the world simultaneously, the condition is termed *pandemic*, as for instance the plague pandemic of 1896 and the influenza pandemic of 1918.

During the interepidemic period, the continuity of infection is maintained by sporadic cases. Equally important in this respect are the mild and atypical cases and carriers. In a similar way the infective agent may persist also in animals which serve as reservoir hosts. The infection in the interval smoulders as it were but breaks out with explosive violence when conditions become again favourable. Whether this epidemic turn is also influenced by any increase in the virulence of the causative bacteria is a difficult problem to settle. The question has been studied with reference to mass infection experimentally caused among lower animals. The results afford little basis for concluding that enhancement of virulence is a determining factor in the causation of epidemics. But it should be remembered that it is not justifiable to apply *in toto* the results of animal experiments to natural epidemics occurring among human beings. The question of natural variation in virulence, therefore, remains still unsettled.

Conditions Governing Infection. The mere lodgement of a pathogenic germ in the body does not lead to infection. The tissues are not a passive source of food supply for the former. One of the general biological attributes of living tissues is the spontaneous protective reaction they put up against a foreign invader. Invasion by an organism is met by reaction and resistance; a favourable issue for the attacking bacterium expresses itself as disease; a favourable issue for the host results in the enhancement of its resistance and in the quiescence or destruction of the invader. Thus, there are in infection two sets of forces operating: the aggressive factors associated with the infecting microbe and the protective factors concerning the host.

Aggressive Factors Pertaining to Bacteria. An important factor concerned with infection is the *virulence* of the micro-organism. It is difficult to give an exact definition of the term virulence as used in relation to bacteria. Virulence means poisonous, but the term is employed in a more comprehensive sense. This property is intimately associated with pathogenicity

but not synonymous with it: several workers regard the term virulence as equivalent to pathogenicity. Virulence is the capacity of a bacterium to multiply in the tissues of a susceptible host and form products which are lethal to the tissues, thus causing disease. A variety of lethal products may be elaborated by the pathogenic bacteria during their growth in tissues as well as in cultures (p. 209). The ability to produce extracellular toxins is sometimes referred to as *toxigenicity*. Only few of the pathogenic bacteria produce such toxins.

Marked variation in virulence is a striking feature of the pathogenic microbes. *Staph. aureus*, for example, is much less virulent than *Str. pyogenes* or the plague bacillus. Besides this interspecies difference, variation in virulence is also encountered among different types of the same species. This is illustrated by the difference in behaviour of the *gravis* and the *mitis* types of diphtheria bacilli or of the bovine and human types of tubercle bacilli towards rabbits. Even different strains of the same species may sometimes vary in virulence; for instance, freshly isolated strains of typhoid bacilli do not always display the same degree of virulence.

We can measure virulence only by its effect on the host. In man the virulence of an organism is judged from the severity of the disease produced and from the case fatality. But for a quantitative study of virulence susceptible animals are usually employed. Virulence is estimated by finding out the minimum dose of the organism (pure culture) that will just kill a susceptible animal—M.L.D. The dose bears an inverse relation to virulence. The minimum lethal dose of toxin is also found out in the same way. The resistance (or susceptibility) of individual animals to the same lethal agent, bacteria or toxin, is subject to appreciable variation and so it will be impossible to lay down an exact minimum lethal dose applicable to all the individuals of any group. Hence it is usual to express the M.L.D. as an average lethal dose for a number of individuals. Virulence is also expressed as a fifty per cent. killing dose or LD 50 dose. Titration of virulence is done on similar animals in groups of equal numbers with graded arbitrary doses. The death rate for each dose is noted and from this the LD 50 dose is calculated. Investigations conducted on these lines have shown that estimating the dose on the basis of a fifty per cent. mortality rate proves more accurate

than on the basis of any other percentage of mortality. Hence the expression LD 50.

Under certain conditions, the virulence of bacteria is found to undergo alteration. It may increase when it is called *exaltation*, or diminish or disappear when it is known as *attenuation* (c.f.). Exaltation of virulence is found to occur by passage through a number of individuals of the same susceptible animal species in rapid succession. The virulence of the rabies virus is thus greatly exalted by serial passage through rabbits. Heightening of virulence by cultural methods is not very successful. Attenuation or a lowering of virulence can be brought about in various ways. Repeated subculturing on artificial media is the commonest method. Other methods are growth in unsuitable media; cultivation at unfavourable temperatures as in the case of Pasteur's anthrax vaccine; growth in media containing weak antiseptics as employed in the creation of the B.C.G. strain; desiccation as exemplified in Pasteur's dry cord antirabic vaccine; passage through insusceptible host species, *e.g.* conversion of the variola virus into the vaccinia virus; and prolonged keeping as in old cultures.

Repeated subculturing and cultivation under unfavourable conditions are productive not only of a diminution or loss of virulence but also of certain associated changes in the cultural characters of a bacterium. These changes comprise bacterial dissociation which is dealt with in another chapter.

Whether fluctuation in virulence occurs naturally in the tissues of the host under the influence of the latter's resistance is not quite clear. That it does is believed by some observers. The conception of *epidemic strain* has its origin in this belief. Some statistical evidence has been produced to show that acute cases of diphtheria are more infectious than carriers. It is held by some workers that during an epidemic a gradual fall in the number of susceptibles in the community equally well as a final lowering of virulence of the invading strain under the influence of increasing herd immunity would account for the termination of epidemics. These matters are still in the speculative stage.

Another property concerned with infection is the *invasiveness* of bacteria. Invasiveness and toxigenicity do not always coexist in the same species. Invasiveness (aggressiveness) is not synonymous with virulence and it is wrong to use so; the former is

employed to denote the capacity to invade and spread into the tissues of the host. A virulent organism need not necessarily be invasive; for example, the diphtheria bacillus is generally very virulent but possesses little power of spreading inward from the focus of infection. Different pathogens have different powers of invasiveness; some tend to remain localised about the point of entry and may or may not spread into the body subsequently, whilst others disseminate rapidly throughout the body. The invasiveness among the toxigenic bacteria seems to bear an inverse ratio to the potency of the extracellular toxins they produce; the most powerful poison is produced by the least invasive one; the tetanus toxin is far more powerful than the streptococcal toxin, but, unlike the latter organism, the former possesses no invasive properties.

The secretion of certain soluble non-specific substances, variously termed *aggressins* or *virulins*, was postulated at one time as the factors responsible for promoting the rapid diffusion of the invading germ into the tissues. These terms have been now given up, as there was no evidence that any such entities apart from the known factors were elaborated. It is a well established fact that bacteria during growth are able to elaborate certain aggressive substances which can destroy many of the tissue components and it is but reasonable to assume that in the mechanism of invasion these aggressive factors are formed and brought into play to reduce the defence forces of the body and prepare the ground. But about the true nature of some at least of these aggressive factors we have now more definite knowledge; such are coagulases, haemolysins, leucocidins, fibrinolysins and spreading factor. Further, it is more than likely that invasiveness does not depend on any one property of the organism but on a multiplicity of properties which work in collusion with one another.

Capsule stands in a separate category from the above factors. It has a morphological existence and is closely allied to the body substance. The capsular material, composed of polysaccharide with occasionally an admixture of nitrogen and amino acids, is not toxic sui generis. Any correlation between capsule formation and virulence has not been established by adequate proof. There is some evidence to show that the virulence of a capsulated pathogen, *e.g.* the pneumococcus, is the maximum when the

capsule is fully developed and that it falls progressively with a fall in the capacity of the organism to produce capsule. It cannot be doubted that the capsule is a protective mechanism of the organism. It is probably anti-phagocytic, in some way counteracting the phagocytic activity of the leucocytes. In this way it may indirectly favour a rapid dissemination of the organism. But the tissues are not helpless against this protective barrier. In their turn, they counter it by developing anti-capsular antibodies which, it is only reasonable to suppose, are meant to break down this bacterial resistance. It is significant that immunisation with the capsular material of the pneumococcus has been found to engender as high a resistance to pneumococcal infection as immunisation with the entire organism.

Dosage. The number of bacteria constituting an infectious dose is no doubt an influencing factor in determining infection. A few organisms gaining entry into the tissue may fail to establish a foothold there against the defence forces of the body. The conception of the M.L.D. is only a further elaboration of this fact. The dose depends upon the virulence of the organism. In some cases, as plague or anthrax, where the virulence of the invading microbe is of a high order, even a few organisms may be enough to set up infection. In the case of weakly virulent organisms, on the other hand, several times this dose will be necessary even to set up a mild infection. There is thus a close correlation between the number of bacteria necessary to produce infection and the virulence of the organism; the greater the virulence the smaller the infectious dose and the minimum lethal dose (M.L.D.).

Selective Localisation: Site of Election. Many of the parasitic bacteria after successful lodgement in the tissues select certain areas remote from the point of entry and colonise there. This is called *selective localisation* and the site so selected the *site of election.* Some, however, remain at or about the point of entry and multiply causing disease. Probably, in these cases the site of election and the point of entry coincide. Or these organisms are constrained to remain at the point of entry due to their limited power of invasiveness. For example, the diphtheria bacillus is confined to its site of entry, usually the tonsils and nasopharynx, where the organism multiplies, causing the disease by virtue of its toxin absorbed into the system. This is apparently

due to its restricted invasive quality. On the other hand, the pneumococcus, a highly invasive organism, apparently finds the lung tissue the most suitable soil for its colonisation. Localisation is also an expression of host resistance, in which case it is not a subjective peculiarity of the parasite. From the primary seat of infection some of the organisms, for example the gonococcus, pneumococcus and others, may subsequently spread into the body and set up secondary foci of infection. Selective localisation after invasion is most strikingly seen in the case of organisms like the meningococcus, the virus of poliomyelitis, the typhoid bacillus, the virus of smallpox, etc., each one choosing a particular tissue in which to set up its activity. Why does one organism prefer one tissue and another organism another tissue is not quite clear. The explanation may have to be sought for in the peculiar biological characters of the invading parasite. Also, some special features in the metabolic processes of the selected tissue of the host, fitting in with the metabolic requirements of the parasite and presenting suitable soil, may have something to do with preferential localisation. Selective affinities are also exhibited by the specific bacterial toxins, as for instance the tetanus toxin on the central nervous system.

Results of Infection. A successful implantation of the organism on the host tissues is not immediately followed by disease. Between the actual entry of the organism and the appearance of symptoms there is an interval which is, within limits, fixed for each bacterium. This is called the *incubation period.* It is a period of silent but intense preparation, rather, intense warfare; the organism multiplies actively and produces poisonous factors in sufficient quantity. These on reaching the susceptible tissues combine with them and injure them and produce derangement of function, giving rise to local or general reaction or both; this is then revealed as disease. The resulting disease, whether local or general, may be either acute, subacute or chronic. When the infection is confined to one part or organ, it is a local infection, *e.g.* abscess, boil, lobar pneumonia, pulmonary tuberculosis. A local infection may subsequently become general. On the other hand, the invading organism may be carried beyond the point of entry by lymphatic or blood stream before starting disease from one or more selected sites or as a generalised infection.

The invasion and persistent presence of bacteria in the blood is called *septicaemia.* It is associated with marked clinical evidence of generalised infection. In the early stages of typhoid fever, very often in the later stages of lobar pneumonia, in osteomyelitis, puerperal sepsis, plague and other conditions, persistent invasion of blood by the causative agent occurs. The active proliferation of organisms in the blood is considered a necessary feature of septicaemia. It is, however, doubtful whether organisms are able to multiply freely in the blood. They may invade the blood in the course of dissemination or as a result of reinvasion from foci of active multiplication set up in the organs at an earlier stage. The term *bacteraemia* is applied to denote a transient presence of bacteria in the blood stream. Many workers use the terms septicaemia and bacteraemia interchangeably. When bacteria from a primary focus of infection invade the blood, they are carried to various organs where they may set up multiple secondary foci of inflammation, necrosis and abscess formation; this condition is known as *pyaemia.* It is the pyogenic organisms that are responsible for this condition. Septic emboli detached from a primary lesion are the usual cause of metastatic abscesses in internal organs. *Toxaemia* is a condition in which the toxic products of bacteria, particularly the soluble toxins, absorbed from the primary seat of infection circulate in the blood and cause damage to the susceptible tissues. Tetanus, diphtheria, cholera and dysentery are some of the notable examples of toxaemia.

Latent Infection and Atypical Infection. Infection may not always result in the production of clinical manifestations. The number of the invading organism may be too few, its virulence low or the resistance of the host high that, although the parasite obtains lodgement in the tissues, it is unable to multiply actively and produce detectable clinical effects. Such a condition is known as *subclinical* or *latent infection.* For example, a syphilitic infection may be concealed from the beginning and remain so for varying periods, sometimes even throughout life. The result of such an infection is, therefore, the establishment of a carrier state. Latency is merely clinical and not pathological. The parasite is not altogether inactive. Minimal pathological changes, too small to cause disturbance of function or clinical symptoms, do occur. Often because of the resistance of the host, the

infection is merely kept under control, establishing a delicate balance between the parasite and the host. At any time the resistance is impaired the condition may flare up into active disease.

In other cases the activity of the parasites, and the consequent response of the tissues, may be sufficiently pronounced to give rise to clinical manifestations. But the symptoms may not at all resemble those characteristic of the particular organism. Such a condition is termed *atypical infection.*

Focal Infection. A circumscribed chronic area of infection in some part of the body constitutes focal infection. Any organism may be responsible for it, but the most important ones seem to be the streptococcus, staphylococcus, gonococcus and *Bact. coli* in that order. The common sites are apices of teeth, tonsillar follicles, accessory nasal sinuses, upper and lower respiratory tracts and the intestinal and genito-urinary tracts. From such primary foci bacteria or their toxic products may be carried through the blood or lymph setting up secondary lesions in the more distant areas, such as the synovial membranes of joints, the sheaths of nerves, muscles and tendons, the eye and other organs. Such undefined non-specific infections are also possibly related to the aetiology of many vague physical and mental disturbances. Absorption of bacterial proteins from the primary foci may sometimes lead to the establishment of a state of sensitisation. Further, a slow process of immunisation may occur, resulting in course of time in the production of a low grade immunity which, however, may not be sufficient to kill off the primary infection.

Mixed Infection and Secondary Infection. An infection is generally due to a single pathogenic bacterium. But sometimes there may be more than one involved from the beginning. It is called *mixed infection.* Examples of this are wound infections, gas gangrene, boils and many respiratory infections. It may be that some one of the organisms in mixed infection is more important than the rest; also, at times the others may be saprophytic as often happens in gas gangrene. When infection by a pathogenic microbe supervenes on an already existing infection, the condition is called *secondary infection*; for example, the secondary invasion of the skin lesions of smallpox by the staphylococcus, the frequent invasion of influenza lesions by the

influenza bacillus, the secondary infection of blood or the urinary tract in enteric fever and other infections. Secondary infection is largely due to the lowering of tissue resistance brought about by the primary invader. Secondary invaders are often weak pathogens usually resident on the host and without a preliminary lowering of resistance incapable of initiating an infection. Secondary infection aggravates the primary condition and tends to prolong it; it may sometimes assume greater importance, even leading to a fatal termination.

Surveying the entire range of bacterial life we see at one end the entirely innocuous autotrophic bacteria and on the other the extremely virulent pathogens, like the plague bacillus, which cause fulminating and fatal infections. Between these can be discerned a slow development of parasitism and pathogenicity. The autotrophs are devoid of any power to infect higher forms of life and are independent of the latter for their metabolic activity; so also the very vast majority of saprophytes. Just a few of the saprophytic bacteria, like *B. subtilis*, may very rarely cause infection; they would seem to develop some aggressive factors as a transient phenomenon. A curious instance is that of a saprophyte *Cl. botulinum* which, while entirely bereft of any ability to invade the tissues, elaborates one of the most deadly poisons known and causes a severe type of intoxication. More advanced in the scale of pathogenicity is another saprophytic soil microbe, *Cl. tetani*, which can just gain a foothold into the body and no further. But by virtue of a powerful toxic factor secreted during growth it causes disease. A step higher than this and belonging to the same category are the gas gangrene bacilli. Then there is a group, represented by the white staphylococci, the pyocyaneus and the colon bacilli, which always live on man, apparently more advanced on the way to establish a peaceful parasite-host equilibrium. Strict parasitism then makes its appearance; the parasitic pathogens have practically no existence apart from the host. The diphtheria bacillus is almost like the tetanus bacillus in its pathogenic status; it has very little invasive powers but produces a powerful exotoxin which is the responsible agent in pathogenesis. But it cannot live independent of the host, except for very short periods. Removed from this and possessing both invasive and toxigenic powers are certain strains of staphylococci and streptococci. Then we come

across a group of parasites which are altogether devoid of toxigenicity but powerfully virulent, for example the pneumococci and the meningococci. Similar in character are the enteric and cholera bacilli. Finally we reach the stage of the highly virulent and invasive organisms, like the anthrax and plague bacilli, against which the tissues are almost helpless to defend themselves.

Bacterial Toxins. How exactly bacteria cause disease is still not fully understood. Injury to tissues cannot result from the mere presence of bacteria. The mechanical effect produced by their presence in blood or other tissues is utterly inadequate to explain the whole train of changes, pathological and clinical, collectively called disease. In order to cause physiological upset it is necessary that the tissue components are damaged or destroyed. Now we know that the pathogenic germs have in their armoury a variety of chemical substances of a poisonous nature which, when brought into contact with susceptible cells, injure or destroy them. These poisonous products are called *bacterial toxins*. It is these toxic products that are in the last analysis responsible for pathogenesis. Besides such toxins, there are other chemical products which, though not toxic themselves, may yet greatly influence the course of infection favourably to the invading parasite.

Bacterial toxins differ markedly in their properties. Accordingly, they are classed into two large categories, namely, *exotoxins* and *endotoxins*. A true toxin is secreted by some of the pathogenic microbes and can be obtained free from the bacterial cell and without damaging it. This type of toxin is, therefore, called the *extracellular toxin* or *exotoxin*. It is also referred to as the *soluble toxin* as it diffuses out of the cell and is soluble in the surrounding substrate. The toxin dissolved in the culture medium is separated from the cell either by centrifugation or filtration. The filtrate constitutes the crude toxin. It is highly toxic, while the residue composed of the cell body is either non-toxic or only weakly toxic. Examples of this type of toxin are those of *Cl. tetani*, *C. diphtheriæ* and *Cl. welchii*.

Most of the pathogenic bacteria do not produce extracellular toxins. In their case the toxic material appears to be the bacterial protoplasm; at any rate, no toxic material can be separated from the cell substance. It can only be obtained by extraction of the bacterial cell or its disintegration. As it does not

come out of the cell body but remains in it, this type of toxin is called *endotoxin*. If suitable fluid cultures of such organisms as the typhoid or the cholera bacillus are filtered, the filtrate is innocuous or only slightly toxic, while the bacillary body, even when dead and disintegrated, is poisonous to animal tissues. Whether there is any separate specific endotoxin associated with such organisms is very doubtful; attempts to isolate such have not so far resulted in any convincing success. In a bacterial lesion of this type, there is the dead and disintegrated bodies of millions of bacteria, and their protoplasm is presumably the toxic factor responsible for pathogenesis. No other poisonous agent has been demonstrated to be responsible for the production of lesions. In striking contrast to it, the most important toxic factor in the case of organisms like the diphtheria or the tetanus bacillus is the soluble toxin, although the dead bacterial protoplasm also may play a minor role.

A great many pathogenic bacteria, such as the enteric, cholera and tubercle bacilli, owe their pathogenicity entirely to endotoxins, no exotoxin being produced by them. In the case of some others, like the diphtheria, tetanus and welchii bacilli, it is predominantly the exotoxin that plays the toxic role and yet in a third group, illustrated by the staphylococcus, streptococcus and Shiga bacillus, both endotoxin and exotoxin are concerned in pathogenesis.

The exotoxins are obtained free of bacteria by centrifugation or filtration through any of the bacterial filters. The endotoxins are obtained by disintegrating the bacterial cells by prolonged grinding, alternate freezing and thawing or by merely allowing them to undergo autolysis. The properties of the soluble toxins are studied by injecting culture filtrates into susceptible animals, but in the case of endotoxins the whole organism itself is injected.

Certain environmental conditions appear essential for the development of exotoxin. For example, the optimum production of the soluble toxins of *Staphylococcus aureus* is obtained in an acid medium; the presence of copper and iron in optimal concentrations appears to be necessary for the synthesis of the diphtheria toxin. No such condition is apparently necessary for the elaboration of endotoxin.

The exotoxins are very unstable compared to the endotoxins. The former soon deteriorate on standing and are readily destroyed

by heat. Most of them are rendered inactive in ten minutes at 80° C.; the botulinum and Dick toxins being exceptions. In contrast to it, the endotoxins are, as a rule, thermostable, withstanding a temperature of 80°–100° C. for one hour. While almost all the exotoxins are subject to proteolytic digestion, the action of such ferments on endotoxins is irregular; a great many are not affected, but some are digested. Again, formaldehyde detoxicates the exotoxins, but it has no such effect on the endotoxins.

When soluble toxins are detoxicated by the action of formaldehyde, the product is called *toxoid* or *anatoxin*. Though toxoid has lost its toxicity, it retains its antigenicity and specific combining powers. This is of very great practical importance in artificial immunisation. Detoxication can also be effected by keeping or by the agency of certain other chemicals.

The extracellular toxins are some of the most powerful toxins known. Only certain plant poisons, for instance ricin and abrin, can approach them in potency. An idea of their potency can be had from the knowledge that, while the M. L. D. of atropine for an adult person is 130 mg., of strychnine 30–40 mg. and of the cobra venom 4·4 mg., the M. L. D. of the crude tetanus toxin is only 0·2 mg. or even less. The botulinum toxin has been computed to be about 10,000 times more poisonous than potassium cyanide. In their purified state the soluble toxins have been found to be lethal to experimental animals in extremely minute doses. For example, a dose of 0·0004 mg. of a purified diphtheria toxin per kilogram of body weight is enough to kill guinea-pigs. The most virulent toxins are those of the tetanus, botulinum and diphtheria bacilli. Compared to the exotoxins, the endotoxins are of very low toxicity. Relatively large doses are necessary to kill experimental animals; for example, a dose of 0·5 c.c. of a killed broth culture of *V. cholerae* is required to kill a standard guinea-pig.

Another important property of the extracellular toxins is their high antigenicity; immunisation of suitable animals with these toxins yield highly potent antitoxic sera. For instance, one cubic centimetre of a diphtheria or tetanus antiserum will contain sufficient antitoxin to neutralise thousands of minimum lethal doses of the corresponding toxin. On the other hand, the endotoxins show relatively poor antigenic capacity and the antisera

prepared against them are generally of low potency, requiring large amounts to neutralise an M. L. D. of endotoxin. Further, the neutralisation of exotoxin by antitoxin follows roughly the law of multiple proportion; that is, if *a* units of antitoxin will neutralise *b* units of toxin, then *na* units of antitoxin will be required to neutralise *nb* units of toxin. No such quantitative relationship governs the neutralisation of endotoxins.

The chemistry of bacterial toxins is only poorly understood, though much attention has been paid to it. None of the soluble toxins has been isolated in an entirely pure form. However, there is sufficient evidence to show that the soluble toxins are proteins composed of the usual amino acids. These toxins, with the exception of the botulinum toxin, are digested by the proteolytic ferments. Hence, administered by the mouth, they are ineffective. How exactly they injure the tissue cell is not known. Some of the endotoxins are proteins, while others appear to be glucolipoids.

In general, the soluble toxins display definite pharmacological properties, resembling in this respect the plant alkaloids. Further, these properties are specific. For example, the tetanus toxin (tetanospasmin) uniformly acts on the motor nerve cells, the diphtheria toxin on the cardiac muscle and adrenals, the toxin of the Shiga bacillus on the intestinal mucosa and so on. In contrast to these, the endotoxins do not evince any characteristic properties. The administration of a lethal dose of any endotoxin gives identical symptomatology to that caused by any other endotoxin, no particular tissue being picked up for selective action in any case.

In this connection, it is interesting to note that certain organic poisons of animal origin display chemical and physio logical properties exactly similar to those of the soluble bacterial toxins; venoms of snakes, scorpions and spiders are well known examples. With these antivenins can be prepared, like antitoxins, in animals, which are specific and of great value.

In addition to the major toxins, bacterial parasites may elaborate during growth many other substances which, though not primarily toxic, are, nevertheless, capable of aggravating infection by virtue of certain special properties they possess. The most familiar of these are *haemolysins* and *leucocidins*, which destroy the red and white blood cells respectively; *coagulases* and

fibrinolysins, which are of enzymic nature; a group of substances called *bacterial X substances* of which the spreading factor is the best known example. Further, the pathogenic bacteria, particularly those that do not form soluble toxins, are associated with certain constituents which sensitise the host tissues; they are termed *bacterial allergens*. Again, partial antigens or *haptens* (p. 234) may be primarily toxic. They may also act detrimentally to the host by combining with non-specific defence factors like opsonins or with specific antibodies. In the pneumococcal infection, for instance, the capsular substance is regarded as interfering with the phagocytic mechanism by combining with the opsonic principles of blood.

Bacterial Haemolysins. These are bacterial products which bring about lysis or dissolution of the red blood cells. Bacterial haemolysins are to be distinguished from the immune haemolysins which are developed by the tissues of an animal in response to injection with the red cells of another species. The former come under the category of bacterial antigens, while the latter belong to the opposite group, bacterial antibodies. In most instances bacterial haemolysins are extracellular and filterable, hence *filterable haemolysins*. Sometimes an organism causes haemolysis on solid media containing whole blood but fail to yield haemolytic factors when grown on liquid media. This has to be remembered when testing for haemolysin.

Many bacterial species form filterable haemolysin. Examples are *Staph. aureus*, certain types of streptococci and *Cl. welchii*; reference may be made to individual organisms. With some species haemolysins are formed early during growth and with others later. Their formation is also conditioned by certain environmental factors. Some are elaborated only in the presence of oxygen, while others are rapidly inactivated by oxygen, *i.e.* oxygen sensitive. The presence of serum in the culture media favours the production of haemolysins. Certain special food factors may also be necessary for their elaboration.

Haemolytic activity is usually demonstrated by a clearing of the medium around colonies grown on blood agar plates. This is the *beta type* of haemolysis. Another is the *alpha type* in which there is a greenish discoloration of the medium in the immediate vicinity of the colonies (see Chapter XVII). A more satisfactory method is by the addition of whole culture or its filtrate to a

suspension of washed red cells in normal saline; incubation of the mixture is followed by disintegration of the corpuscles and liberation of haemoglobin.

Bacterial haemolysins belong to the category of exotoxins. They are thermolabile, being inactivated by heat at 55° C. for thirty minutes. Some are oxygen stable; some are oxygen sensitive and are capable of remaining in the reduced as well as in the oxidised state. Bacterial haemolysins are probably protein in nature. They are antigenic and stimulate the production of antihaemolysins.

Haemolysins are not simple entities; there are several types of haemolysins, exhibiting differences in immunological specificity, resistance, the species of erythrocytes on which they act and so on. Even a single bacterial species may produce more than one haemolysin; for instance, some strains of streptococci produce two haemolysins, one oxygen stable but heat sensitive and the other heat stable but oxygen sensitive. The haemolytic activity is determined by the source of the erythrocytes; that is, different haemolysins act on erythrocytes of different species of animals; a particular haemolysin may lyse rabbit cells but not sheep cells. For example, certain strains of staphylococci produce at least two haemolysins, one acting on both rabbit and sheep cells and the other acting only on sheep cells.

What part haemolysin plays in the pathogenesis of infection is not quite clear. There is no certain evidence of in vivo haemolysis taking place in all cases of infection with haemolytic pathogens. The haemolytic activity, however, appears in some cases at least related to virulence, as in the case of streptococci and staphylococci. On the other hand, the capacity to produce haemolysin is not peculiar to the pathogenic species; many saprophytic bacteria produce filterable haemolysins.

Leucocidins. Leucocidins constitute another type of aggressive factors formed by some bacteria during growth. They should not be confused with the immune leucocidins which can be prepared in the experimental animals by injecting them with foreign leucocytes. Their action is principally on the polymorphonuclear leucocytes and consists of degenerative changes, fragmentation of the nuclei and death with or without disintegration.

Many species of bacteria, such as staphylococci, streptococci and pneumococci, form leucocidins. As in the case of

haemolysins, there are many leucocidins. The same species, nay even a single strain, may elaborate more than one leucocidin. Again, not only between different species of bacteria but also between different strains of the same species are encountered wide differences in the quantities of leucocidins produced. Leucocidins differ in their resistance to heat; they also exhibit a selective action on the leucocytes of different animal species.

Leucocidins, like haemolysins, are antigenic and bear a close resemblance to them. Some of the haemolysins also exhibit leucocidal properties, suggesting that both are identical. What part leucocidins play in the pathogenesis of infection has not been fully elucidated. Probably, they help the organism in overcoming the defence mechanism of the host tissues and thereby pave the way for the rapid spread of the invader from the point of attack.

Coagulase and Fibrinolysin. Some bacteria, notably staphylococcus, produce a ferment-like substance, *coagulase*, which accelarates the clotting of human and rabbit plasma. The addition of the particular organism to citrated or oxalated plasma leads to the coagulation of the latter after a specified time interval. The coagulase is relatively heat-resistant; exposure to 100° C. for thirty minutes fails to inactivate it completely. It is not filterable. Available evidence suggests that there is protein in its constitution. Coagulase is not definitely known to be antigenic. Its role in the mechanism of infection is by no means clear. Coagulase may be a protective factor, enabling bacteria to consolidate the ground gained during infection.

Fibrinolysin is another ferment-like substance exhibiting just the opposite property, *viz.*, dissolving the blood clots whether formed through coagulase action or other agencies. The capacity to produce fibrinolysin is associated with many pathogenic microbes but particularly with certain groups of streptococci. To some extent fibrinolysin is specific; for instance, certain strains of streptococci will dissolve clotted human plasma but not clotted animal plasma. The same bacterial species may produce both coagulase and fibrinolysin. Though enzyme-like, fibrinolysin is relatively heat stable. It is probably protein in nature and antigenic. Because of its lytic action on clotted plasma, it is only reasonable to suppose that fibrinolysin has a greater role in the mechanism of infection than coagulase. By dissolving

the fibrin barrier erected by the host defence against the invading organism, apparently to localise the infective process, fibrinolysin may facilitate rapid diffusion of infection into the tissues, thus promoting invasiveness. In favour of this view is the fact that organisms that form fibrinolysin are markedly invasive, although the opposite is not always the case.

Spreading Factor. Another interesting substance that is developed by many pathogenic bacteria is what is called the *spreading* or *diffusing* factor. It probably belongs to the category of bacterial products described under the name of bacterial X substances which appear to be capillary poisons. It markedly enhances the permeability of tissues. Extracts of organisms that elaborate the spreading factor, when combined with other bacteria, toxins or materials like Indian ink and injected into the skin, promote the rapid diffusion of these into the tissues. Many pathogenic bacteria produce this factor, particularly the invasive ones like certain strains of staphylococci, streptococci and pneumococci. There can be no reasonable doubt, therefore, that the capacity to produce this factor is closely related to invasiveness. An interesting point is that a similar factor which increases the permeability of tissues has been extracted from certain mammalian tissues, notably the testes. There is also evidence to suggest that the spreading factor is identical with the enzyme hyaluronidase.

We have indicated above the various well defined weapons of aggression and defence possessed by the pathogenic microbes, by the aid of which they invade the living tissues, overcome resistance, establish infection and cause disease. Yet there may be others of minor importance, of which we have only faint knowledge, which go to constitute the totality of bacterial virulence, and may, therefore, be taking part in the process of infection. It is not known what exact part the necrotoxin of staphylococci plays in natural infection. More obscure is the nature as well as the role of the hypothermic factor reported in association with the Shiga bacillus. Another interesting observation is the change in the level of blood sugar brought about by certain intestinal pathogens in the experimental animal. Also, little is known about the function or purpose of some of the minor antigens described in association with many pathogenic bacteria. These and similar factors demand intense study before their actual role, if any-

in the mechanism of infection and pathogenesis can be fully revealed.

Ptomaines are not true bacterial poisons and are not derived from bacteria. They are protein cleavage productas of the nature of amines produced as the result of bacterial activity on the substrate. As they were considered to be toxic, these amines received the name ptomaines. Ptomaine poisoning is an exceedingly rare event and what was previously regarded as ptomaine poisoning was only true bacterial food poisoning caused by the consumption of food containing living organisms or their toxins.

Protective Factors of the Host. Successful infection is also largely dependent on the protective powers of the host. Broadly speaking, all these powers may be included in the term immunity which means resistance to infection, inherited or developed during life. The host is provided with different methods of defence some of which are non-specific and common to all animals and operable against any parasitic species. All these factors of resistance can be brought under four categories, mechanical, chemical, cellular and humoral. It is possible that certain non-specific reactions of the body may also have a defence significance. For example, there is the view that fever itself may enhance the resistance of the host against the invading bacteria. The first line of defence against microbic invasion is formed by the mechanical obstruction offered by the investing layers of the body. This is reinforced in no small measure by their secretions and excretions which are also concerned with preventing the organism from entering into the body. The cellular and humoral defences come into play only after the invading microbe has actually reached the tissues.

Mechanical Barrier to Infection. This is provided by the natural coverings of the body surfaces, the skin and the mucous membranes with their appendages. The vast majority of micro-organisms are unable to penetrate the intact surfaces. Even those organisms that are normally present in the superficial layers of the skin are unable to enter the subjacent tissues unless it is favoured by some cutaneous injury, such as a cut or burn. The ducts of the sweat glands and the hair follicles do not usually permit the entry of these organisms without some sort of previous trauma or dirt accumulation. Available evidence also tends to show that the clean skin may be actively bactericidal; organisms that are

not normally found on the skin, for instance the typhoid and colon bacilli, are destroyed within a short time when swabbed on the skin. Appendages like hair and the cilia of the epithelium are further hindrances to the entrance of bacteria, especially when they line narrow passages as those in the respiratory tract. For instance, bacteria that are present in the inspired air are largely filtered off during their passage through the upper respiratory tract; many of them are also caught on the moist surfaces of the mucous membrane and the ultimate result is that few reach the smaller tubes.

Chemical Action of Secretions and Excretions. In virtue of their bactericidal properties, these tend to destroy the invading parasite. In addition, they also exert a mechanical or flushing action and remove the bacteria from the point of attack. The flushing action of tears is a common experience. As already mentioned, many of the particulate materials including germs entering through the nose are caught in the mucus and ejected out periodically. It has been shown recently that a virus inactivating agent (VIA), unconnected with lysozyme, is normally present in the nasal secretions. Its role in resistance to infection has not been worked out. Mucus, wherever it is secreted, fulfils the same protective function; it has no bactericidal quality. Saliva is only mildly bactericidal and its anti-infection role is almost wholly mechanical in nature. The free hydrochloric acid of the gastric juice is highly bactericidal and acts as a hindrance to the passage of most bacteria through the stomach. The normal vaginal secretion is acid and displays marked bactericidal properties. Sweat and sebum serve as mere physical barriers against the attack of bacteria. The antiseptic properties of most of the secretions which are not of a high order, appear to be due to the presence of a ferment-like substance called *lysozyme*. Although its bactericidal power against certain saprophytes is high, its effect on the pathogenic forms is not appreciable. The greatest concentration of it is found in tears, whereas it is not present in sweat, urine and cerebrospinal fluid. It is also found in all tissues and also in egg white. The pH of the secretions is also an important factor in determining their bactericidal efficiency.

Normal Bacterial Flora. The normal bacterial flora (p. 163) of the body would also seem to play a protective role. Probably by means of the normal products of growth, such as

acid, the indigenous bacterial population of a region may prevent colonisation of that region by an exotic species. For example, the aciduric bacteria of the intestines tend to prevent the colonisation of the gut by the abnormal pathogenic bacteria; Doderlein's bacillus of the vagina also acts likewise.

Cellular Activity or Phagocytosis. The capacity of certain types of cells to engulf and destroy bacteria is a non-specific but important factor in the natural defence mechanism of the body. The cells of the reticulo-endothelial system and the leucocytes, particularly the polymorphonuclear type, are the ones mainly concerned with phagocytosis. Phagocytosis is by no means an independent action of the cells; certain natural substances, and even antibodies, contained in the body fluids exercise a profound influence on phagocytosis. The subject is discussed in detail in a later chapter.

Humoral Activity. The tissues manufacture antibodies against the invading parasite and throw them into circulation. Thus, the fluids are able to exert a powerful protective action against infection. These antibodies may destroy or help to destroy bacteria or to neutralise the bacterial toxins. Infection serves as a stimulus for their production and the degree of response is an essential factor in determining the course of disease (p. 222).

General Factors. Besides the above protective mechanisms, certain conditions concerning the host and his environment do play a significant part in determining not only infection but also its subsequent course. Species, racial and individual differences in immunity are discussed in a following chapter. The effect of the *age* of the host on resistance to infection is not uniformly conspicuous in all infections; in some it is pronounced, while in others inapparent. In general, the immature tissues would seem less able to cope with infection than the mature. It has been demonstrated that the young infant responds but feebly to active immunisation. There is some evidence to show that its reticulo-endothelial system does not react well to the stimulus of infection. A subnormal titre of complement is a notable deficiency of the new born; similarly, some workers have postulated the existence of a special antibody-forming apparatus which is immature in the infant but becomes mature with growth. This, however, is based on slender evidence. But it is a well known observation that infants up to six months or a year are relatively

immune to many infections. What gives this immunity is the passive transfer of antibodies from the mother and when this wears out, as it soon does, the infant is left with little or no resistance. The subsequent years are the most susceptible period. The increased resistance to infection of the grown-up individual can likewise be accounted for, in part at least, by the presence of antibodies in the blood formed as a result of subclinical infection. At the other end of life, consequent on several degenerative changes, the powers of resistance are on the wane and the aged are only too easy victims of many infectious diseases. A *sex* difference is observable in the incidence of several infectious diseases. For example, males suffer more frequently than females from pneumonia, meningococcal meningitis, syphilis, gonorrhoea and others. Whether there is any hereditary basis for this disparity or whether it is entirely a reflection of greater oppportunities should remain subjudice until more work is done on the subject.

Climate and *season* are factors that exercise marked influence on the incidence and mortality of many infectious diseases. It is a well attested fact that the respiratory infections are more frequent, more acute and more frequently fatal in the temperate regions than in the tropics, whereas the alimentary infections are more common and more serious in the tropics. Yellow fever, a very common disease in certain tropical and subtropical regions, is absent in the temperate regions. Similarly, with many infections there is a well defined seasonal distribution. The greater incidence of cholera and typhoid fever during the months of July to November in many parts of this country is a common observation. Both climate and season may operate through their effect on insect life; the yellow fever mosquito does not thrive in the higher latitudes and the fly population is highest during certain seasons. They may act by inducing closer association among people or of people and animals, which affords greater opportunities for transmission of the infective agents. Among people forced by poor housing conditions or inclemency of weather to spend long hours in overcrowded and ill ventilated dwellings, respiratory and other infections transmitted directly from man to man are more frequent than among those who live under better conditions. Again, bubonic plague is commoner during cold weather, partly because the close association of rats with man

becomes closer during this period. Whether temperature or season by itself has any stimulating or inhibiting effect on tissue resistance is at present little more than conjecture.

The health of the individual exerts a tremendous influence in determining the incidence as well as the course of infection. The physiological well-being is influenced by a variety of factors. Of these, *food* is the most important. Improper and inadequate food impairs the general vitality and resistance in no small degree. Vitamin deficiency, particularly of vitamin A, is an important predisposing cause of bacterial infections. Protein deficiency also appears to have a similar effect. Malnutrition and starvation, which are only too common in vast areas of the world, are the closest allies of infection and the greatest enemies of the public health worker and the clinician. Proper *raiment* and proper *housing* are also closely related to general well-being. Other factors that interfere with the general resistance of the individual are pre-existing *diseases*, *alcoholism*, certain *occupations*. *overexertion* and *fatigue*. All these are, therefore, predisposing factors of infection.

The foregoing considerations make it abundantly clear that successful infection, and to a less extent its subsequent course, is the final outcome of the interplay of a variety of factors relating to the parasite, the host and his environment.

CHAPTER XI

IMMUNITY

Immunity is the capacity to resist infection. The opposite condition is called susceptibility. It is self-evident that these two conditions are inversely related to each other. Anything that enhances the resistance of a host to infection reduces his susceptibility and *vice versa*. Broadly speaking, immunity to bacterial infection exists either as innate or genetic powers, transmitted from parents to progeny and entirely independent of external conditions for their origin and functioning, or as acquired properties which depend upon environmental factors, such as contact with infection, for their production. It should be clearly understood that, while this division is convenient for discussion, it is often impossible to assign a particular case of immunity to natural resistance or to resistance acquired as the result of inapparent infection. Further, experience teaches that resistance to infection, in whatever form it is seen, is a variable factor. Different host species and even different individuals of the same host species display different degrees of resistance to the same bacterial species. It is a common observation that when an individual has once suffered from smallpox or measles, he does not generally get it again in future, indicating the acquisition of life-long protection as the result of the attack, while even the closely associating domestic animals, like cats and dogs, do not contract the infection at all. Again, an individual may react fatally to an infection, another with a serious or mild disease, while a third may show no apparent reaction at all. All these clearly indicate that immunity exists under different conditions and in different degrees. The various types of immunity are summarised below:

1.	Innate Immunity	Natural	
2.	Acquired Immunity	(*a*) Active	Naturally acquired. Artificially acquired.
		(*b*) Passive	Naturally acquired (congenital). Artificially acquired.

Innate Immunity. Resistance to infection is a natural gift; it may be complete or partial with varying grades. This natural non-susceptibility to infection is called *innate immunity*. While innate immunity is a common phenomenon, we know practically nothing about its immunological basis. Though much experimental work has been done on this subject, the results are quite inconclusive. Immunity, or susceptibility, shows species, racial and individual variations. Though man and animals are alike subject to infectious diseases, the type of infection is not always common to them. Syphilis, gonorrhoea, leprosy, measles, diphtheria, cholera and many other diseases do not occur spontaneously in any of the lower animals and it is even difficult to reproduce many of them in any of the animals; so also birds are immune to tetanus a disease affecting man and certain of the lower animals. Conversely, certain diseases are peculiar to animals and are not found to affect man, *e.g.* rinderpest or chicken cholera. These examples fully illustrate *species immunity*.

Again, all races of the same species are not found to exhibit the same proneness to a particular infection. For example, anthrax is a common disease of sheep, but the Algerian race of sheep is singularly immune from anthrax. This is an instance of *racial immunity*. It is very doubtful whether racial immunity is a striking feature among human beings. Many attempts have been made to establish its existence. The results of most of them are of questionable evidential value. The oft-quoted resistance of the Negroes to yellow fever has been all but completely proved to be the result of inapparent or mild childhood infection. The greater incidence of tuberculosis among the American Negroes can be traced on closer scrutiny to unfavourable environmental factors as poor diet, poor housing conditions and the like. Nor is the observation that some of the primitive races are extraordinarily susceptible to certain infections like tuberculosis and syphilis when first introduced among them a weighty support for the existence of any special racial susceptibility among them. For history tells that the Europeans also reacted to syphilis in a similar manner when it was first introduced into that country. Thus, most of the instances quoted of racial immunity among man can be controverted on equally or often more reasonable grounds.

Individuals also evince wide differences in their capacity to resist infection. It is a common observation that all those who

are exposed to a particular infectious disease do not get the infection; all those who come in contact with a diphtheria patient or a meningitis patient, for example, are not attacked by the disease; some of them are apparently unaffected, some resist it for a long time, while a few may readily imbibe the infection. Of those who develop the disease, some get it in a fatal form, many in a serious form and recover only after a prolonged course, while some develop only a mild or abortive form. Clearly then, uniformity of reaction on the part of individuals is lacking. The basis of this difference, no doubt, is the variable factor of *individual resistance*. The ultimate entity that is responsible for these variations in immunity is the living tissue. Therefore, it is easy to understand that tissue resistance is also a variable factor.

On what exactly does individual resistance depend is not clear. There may not be much doubt that some innate protective mechanism is inherited by the individual; but regarding its fundamental nature, component parts or mode of operation little is known. We have to fall back upon such factors as the bactericidal property of tissues and body fluids, opsonins, complement and probably other unknown entities. As also in the case of species resistance, it is a plausible stand to argue on Ehrlich's hypothesis that the tissue units of the resistant individuals lack the special receptors which are ultimately essential to bring about effective contact between bacteria or their products on the one hand and cells on the other. There is, however, no doubt that resistance in a great many cases of supposed innate individual immunity rests on immunity acquired through inapparent contact with the infectious agents. More often than not antibodies, the so-called natural antibodies, are present in them and most probably that provides the clue to their immunity.

Acquired Immunity. The resistance developed as the result of accidental infection or artificial immunisation is called *acquired immunity*. It may be *active* or *passive*. The term active is used to denote that the immunity is set up by the unaided activity of the tissues of the host, no extraneous help being involved in its production. Passive immunity, on the other hand, indicates that the tissues of the beneficiary of the immunity play but a passive role in the development of that immunity; it is transferred from an actively immunised individual to a non-protected one.

The development of protection after an attack of infectious disease is an ancient observation, though its rational basis is of recent unfolding. Many of the infectious diseases seldom occur more than once in the same individual. Such is the case with many of the virus diseases, typhoid fever, plague, diphtheria and others. The protection conferred by a single attack of any of these diseases is lasting, often for life. Should a second attack occur, it will be only of a very mild or abortive form. In some diseases, however, though immunity develops, it is evanescent or short-lived as in syphilis, pneumonia, gonorrhoea, influenza and others. The above are instances of acquired immunity developed as the result of natural or spontaneous infection. In this category should also be included the resistance developed as the result of latent infection or carrier state, although it may be but of a low degree.

It has been found possible to imitate nature and create or enhance immunity artificially by injecting bacteria or their products (exotoxins) into the susceptible man or animal. In this, similar to what happens in natural infection, we are artificially introducing into the tissues the infectious agent, bacteria, or its toxins and thus provide the stimulus necessary for producing immunity without causing disease. This is acquired artificial immunity. In both the above types of acquired immunity, the condition is created solely through the efforts of the host tissues and, therefore, they come under the category of acquired active immunity.

The method of artificial immunisation by the injection of dead or living organisms is called *vaccination*. It is done in a variety of ways: with sublethal doses of living virulent organisms, this method is not now practised in man; with attenuated living organisms (p. 202) as in anthrax, B.C.G. or smallpox vaccination; or with dead bacteria, as in immunisation against staphylococcal or colon bacillus infections. There is evidence to show that vaccination had been practised (China and India) in very ancient times. However, Edward Jenner was the first to discover and demonstrate the value of vaccination on a scientific basis and inaugurate a rational method of immunisation against smallpox (1796). Many before Jenner had noticed that milk maids and other dairy workers, who got vaccinia or cowpox in the course of their work, escaped getting smallpox when the latter prevailed in the community. But Jenner was the first to recognise that the

pox so developed was identical with the lesions of smallpox. This observation led him eventually to the discovery that injection of cowpox material into a susceptible man conferred protection against smallpox. The next notable contribution to the subject was that of Louis Pasteur. As the result of extensive experiments on chicken cholera, anthrax and rabies, Pasteur, in the second half of the last century, established the principles of vaccination on incontrovertible scientific basis. In all his work Pasteur employed only living organisms, though attenuated of virulence. Jenner's as well as Pasteur's methods of attenuation have undergone many technical modifications, though the underlying principle remains the same. Pasteur's method of antirabic immunisation is still in vogue in France and some other parts of the world. Yellow fever and tuberculosis are other diseases in which vaccination with attenuated organisms has been introduced as a measure of prevention.

Active immunisation with dead bacteria was of a later introduction. Vaccine prepared by this method is now more commonly employed than attenuated vaccine in treatment as well as for prophylactic immunisation in man. Cholera, enteric fever and plague are some of the diseases against which prophylactic immunisation with dead organisms is now widely practised. An important point to bear in mind in connection with this method of immunisation is that bacteria undergo spontaneous variation into avirulent form and vaccines made with such antigenically degenerate forms will not confer efficient protection. Hence, organisms in the virulent or smooth state only should be employed in the preparation of vaccines. A no less important point is that in the killing of bacteria only those methods that will cause the minimum change in the physicochemical state of the antigen should be employed, lest destruction of any of the vital components, that may otherwise occur, may interfere with the efficiency of immunisation. The methods of killing in common use are heating at low temperature, 55°–60° C. for thirty minutes, and treating with certain chemicals such as formalin —0·1 per cent., phenol —0·5 per cent., and tricresol —0·3 per cent. Treatment with alcohol has been recommended of late as it does not destroy the labile antigen.

Active immunity can also be induced artificially by injecting with soluble toxins. It is called antitoxic immunity as against

antibacterial immunity produced by vaccination. Active artificial immunisation with toxin is founded on the observation of Behring and Kitasato who successfully immunised rabbits against diphtheria by injecting them with filtrates of diphtheria culture. Since then, much work has been done on the subject and immunisation has been shown possible in all cases in which a true exotoxin is formed. This method of immunisation is now extensively practised, notably in the prophylaxis of diphtheria and tetanus. It is also employed in immunising animals for the production of immune sera. An important advance in this line has been the introduction of toxoid, a detoxicated but antigenic product, as an effective substitute for the risky toxin.

The duration of active immunity induced by artificial means, be it by vaccination or by the injection of soluble toxins, may vary from a few months to several years. It may not be so efficient as that engendered by disease. The reason for this may not be far to seek. In the former the stimulus provided is far more restricted than that provided by actual disease in which the stimulus is effective practically throughout the body. Again, the immunity produced by artificial means may vary in degree contingent on the quality of the antigen employed. Some bacteria, like the typhoid bacillus and the staphylococcus, form efficient antigens, while others, like the gonococcus, exhibit but poor antigenic virtue. The practical value of active artificial immunisation is dealt with under individual organisms.

Local Immunity. The hypothesis of local immunity has been chiefly advocated in recent years by Besredka. He believes that specific localisation of tissues by bacteria is an essential factor in the production of disease. By whatever route an organism gains access to the body, eventually it should reach the tissue of its choice before it can cause disease. If, therefore, the particular susceptible tissue is actively immunised against the specific agent, the body as a whole is thereby protected against the disease. For example, according to Besredka skin is the only susceptible tissue to anthrax and if that is rendered immune by active artificial means effective protection of the body against anthrax can be ensured. If the bacilli were to gain entry through any other route, they will be rapidly destroyed by phagocytes. Similarly, for intestinal pathogens intestinal mucosa is the vulnerable tissue and active immunisation of the latter should

be the rational mode of safeguarding against infection by the former. There is no doubt that a certain amount of immunity is produced by this method. But there is no proof that it is due only to a local stimulation in Besredka's sense and not to a general stimulation caused by the antigen absorbed from the site of application. The treatment of various skin conditions due to staphylococci and streptococci by *antivirus* (sterile culture filtrates of these organisms) and the prophylaxis of intestinal infections by the use of bilivaccines are meant to induce local resistance. Besredka's views have failed to achieve general acceptance.

Passive Immunity. In this type of immunity protection is imparted from outside and the tissues of the protected animal do not participate in its production. Hence the term *passive immunity*. Behring and Kitasato were the pioneers in the field of artificial passive immunity. They found that after injection of rabbits with tetanus toxin or diphtheria toxin certain substances possessing the power of neutralising the toxin were present in the blood of the animal. Such substances they called *antitoxins*. Further, they showed that the serum of an actively immunised animal possessed specific protective and curative powers. These facts are now well established. The sera of animals actively immunised with bacteria or their toxins are known as *immune sera* or *antisera;* these owe their effect to the specific immune bodies or antibodies present in them. The administration of such sera confers temporary immunity to the recipient. Obviously, in this type of immunity the tissues of the host play only a passive role; the body is merely the recipient of antibodies produced by some other host. Passive immunity is often inherited by the progeny, the immune substances passing by the transplacental route or after birth through mother's milk. Immunity so derived is of short duration and the infant is protected at the most for six months to a year.

In active immunisation a certain time interval is necessary for the elaboration of protective substances by the cells of the host. This method is, therefore, not available for the clinician in a declared acute disease where the question of time is an important consideration. The value of artificial active immunity lies more in prophylaxis than in treatment. The protection created by active immunisation is also of much longer duration

than that by passive immunisation. On the other hand, in passive immunisation there is a readily available method of conferring immediate protection. But its effect is shortlived. The maximum concentration of immune serum in the blood of the recipient, when administered by the subcutaneous or intramuscular route, is attained in two to three days after injection. This becomes less and less and the serum disappears altogether from circulation within three weeks; the globulins of horse serum tend to be eliminated from circulation as a foreign body.

Immune sera are classified as *antitoxic sera* and *antibacterial sera.* The former is obtained from animals actively immunised with toxins and the protective substances contained in them are called *antitoxins.* The diphtheria antitoxin and tetanus antitoxin are well known examples of this type of antisera. Antibacterial sera are prepared from animals by immunising them with bacteria; the antibodies so generated are directed against the homologous bacterial cell, *antibacterial antibodies.* Examples of this type of antisera are the antiplague serum, antimeningitic serum and antityphoid serum. Antitoxic sera exert a far greater beneficial effect than antibacterial sera and have, therefore, obtained much wider application in prophylaxis as well as in treatment. Although of limited value, antibacterial sera are also employed prophylactically, as for example, administration of antiplague serum to the antiplague staff. They are also used for curative purposes, as in the case of meningitis and lobar pneumonia due to Type I pneumococcus. Antibacterial immunity may be induced also by the administration of convalescent serum obtained from persons recovered from the particular disease. Being homologous, it may remain longer in circulation than horse serum, thus prolonging the duration of immunity.

The capacity to produce antibodies under certain conditions is a general biological attribute of living animal tissues. Similarly, the capacity to incite the production of antibodies is not confined to bacteria and their toxins. Non-bacterial chemical poisons, such as ricin and abrin of plant origin, were shown by Ehrlich to be capable of provoking antibody production. Numerous other workers have demonstrated the same capacity in the case of foreign cells, *e.g.* red blood corpuscles and spermatozoa (cytolysins); serum proteins; milk; egg albumin; organic poisons, like snake venom and scorpion poison. The

development of antibodies has been noted in certain metazoan infections. But it is not known whether they have any protective or curative function.

Mechanism of Resistance. Apart from the phagocytic activity, the essential mechanism whereby immunity is established by the host against bacterial infection is by the production of antibodies. These are present in the blood and other body fluids. Gross changes in the chemical make-up of the body fluids of the immunised individuals are not discoverable. Their presence can, however, be demonstrated both by the in vitro and in vivo reactions. It has to be noted that in the protective process certain natural substances found in the blood, such as complement and opsonins, act in conjunction with the antibodies. The nature of the antibodies differs very materially according as the invading organism produces exotoxin or only endotoxin. In the former case antitoxic antibody, which acts by neutralising the specific toxin, is produced in the body. It should be noted that in infection by an organism which produces extracellular toxin, the mechanism of resistance does not solely consist of the formation of neutralising antitoxin which is powerless against the bacterial cell; antibacterial antibodies and phagocytes also take part by destroying the invading bacterium. Where infection is due to organisms which produce no demonstrable exotoxin, resistance is probably due to the production of antibacterial antibodies which, acting on the invading bacterium in a variety of ways, help to destroy them. As a matter of fact, the final eradication of all invading organisms is essentially a function of antibacterial immunity. The development of hypersensitiveness as the result of bacterial invasion is believed to be yet another factor towards immunity production.

Thus, the chief factors concerned in the mechanism of resistance against infection are the tissue cells, which are concerned with the destruction of bacteria by phagocytosis, and the antibodies, or immune bodies, contained in the blood and body fluids, which form the chemical antidote against the bacterial parasite and its toxin. It must be clearly understood that these are not mutually exclusive but closely interrelated. In the early days of immunology, two different schools of thought existed regarding the question of the mechanism of immunity. Following the teaching of Metchnikoff, one school considered phagocytosis

as the supreme factor concerned in resistance; this constituted the *cellular hypothesis*. Metchnikoff regarded complement, opsonins and other similar factors as derived from the effete and broken down leucocytes and therefore all protective reactions as traceable in the ultimate analysis to cells. The other school maintained that antibodies formed the fundamental factor of defenec against bacterial infection. This was the *humoral hypothesis* and Ehrlich was its chief proponent. It is now generally accepted that both factors are intimately concerned with the defence of the body.

Antigens. A substance which incites the tissues to produce antibodies is called an *antigen*. All substances do not possess this property. Before they can be categorised as antigens, they should satisfy certain criteria. An important condition is that they should be foreign to the animal generating the antibodies; otherwise, the tissues will not be stimulated to produce antisubstances. Sheep's erythrocytes, for instance, will not incite the production of antibodies when injected into another sheep, but when injected into a rabbit evoke a vigorous antibody response. But all foreign substances are not antigenic; for instance, sugar, quinine or salvarsan does not exhibit antigenic activity. Foreign substances, in order to be antigenic, must be protein in nature. Further, all foreign proteins are not antigenically active; for example, gelatin, though a protein, is not antigenic. The antigenic property of a foreign protein depends on its chemical constitution. The exact nature of this chemical constitution is not clearly known, but there is some evidence to show that a protein, in order to be effectively antigenic, should contain aromatic acids, *i.e.* acids containing benzene rings.

In general, antigens are colloidal in nature composed of large molecules. On hydrolysis of the antigenic proteins, reduction in the molecular size occurs and with it loss of antigenic capacity. Hence, the size of the molecule may be an important determinant factor of antigenicity. Antigens must be soluble in the plasma of the animal, as, otherwise, it cannot reach the generating tissue. Another important point is the channel through which the foreign protein is introduced into the body. If it is given per mouth, it is digested and reduced to smaller units, in which state it ceases to function as an antigen. For this reason, antigen should be administered parenterally so as to bring it into contact with tissues unchanged.

Another characteristic trait of an antigen, apart from its ability to produce antibodies, is its specific combining capacity. In other words, not only will an antigen stimulate the production of antibodies, but it will combine, both in vivo and in vitro, with the antibody so produced; it will not combine with any other antibody, except those formed against closely related antigens. An example of the latter is the cross reaction shown by human serum with the antibodies prepared against the serum proteins of the anthropoid apes and certain monkeys. The specific union of antigen with the corresponding antibody takes place not in a random fashion but follows certain definite laws (p. 212).

Specificity of Antigens. There is ample evidence to show that the basis of immunological specificity is chemical and not biological. It has been pointed out in an earlier section that, besides bacterial antigens, a variety of other substances possess the property of antigenicity, *e.g.* red blood corpuscles, serum, egg white, milk, certain vegetable toxins, snake venom and others. An outstanding characteristic of all such antigens is their specificity; this means that the antibodies produced by a particular antigen combine only with that antigen or other closely related antigens and not with any other. It is now established that specificity is in the last analysis founded on chemical composition and not merely on biological position. The large complex protein molecules are characterised by the presence of certain atomic groupings on their surface. The chemical composition of these active groupings and their spatial relationship in the molecule are the determining factors of specificity. It is these subtle differences in the intimate chemical structure of the antigenic molecules that make it possible to differentiate by immunological means between so closely similar substances like the egg albumin of the duck and of the hen, the haemoglobins of different animal species and different plant proteins.

Though specificity is a function of the chemical structure of the antigen molecules, it is, nevertheless, a striking feature of the biological species. This, of course, is due to the fact that in nature there is a common distribution of chemically identical protein matter among members of the same biological species. That is why specificity is seen to exist characteristically among the same species—*species specificity*. The presence of antigenic fractions with close structural similarity will explain the pheno-

menon of cross reaction not infrequently encountered between allied biological groups, as well illustrated by the cross reaction between the serum proteins of man and monkeys. Again, while it is true that a serological relationship usually points to a biological relationship, this is by no means universal. For, a random distribution of antigenic groupings having chemical identity may also be encountered in widely different biological species. This would seem to account for such apparently non-specific reactions like the Weil-Felix reaction.

The study of artificially prepared antigens or synthetic antigens has greatly elucidated many of the problems connected with the phenomenon of immunological specificity. From these studies it has been found that slight changes brought about in the structure of antigenic proteins, such as the introduction of new atoms or groups, like iodine, nitrate and others, lead to large modifications in their immunological behaviour. Thus, a serum iodised with Lugol's iodine produces on injection into the rabbit an immune serum which does not react with the original unmodified serum but readily does so not only with the modified serum, but with similarly treated sera of other animal species, showing thereby that iodisation has profoundly altered the specific nature of the serum. Further, it has been shown that striking changes in specificity may likewise be brought about by esterification, methylation or acetylation of antigens. And it is interesting to note that these changes do not involve any interference with the basic aromatic radicals but only with the salt-forming groups of the protein molecule. Furthermore, heating, partial denaturation and other similar processes induce large changes in the immunological specificity of antigens. For example, heated rabbit serum, which is still antigenic, is different in this respect from the unheated serum. The former behaves as a foreign protein in the rabbit and stimulates the production of antibodies which, however, fail to react with the unheated serum, although reacting with the heated serum. Such heated antigens are called *cactoproteins*. Investigations on the above lines indicate that not only is the nature of the active grouping or groupings of supreme significance in determining specificity, but also its position, ortho, meta or para, within the antigenic molecule; any alteration in it will be productive of marked changes in specificity.

Another interesting observation is what is often referred to as *organ specificity*. Certain organs of mammals are found to contain identical antigens. For example, the lens substance is immunologically the same in all mammals but is antigenically dissimilar from other proteins of the body; similarly, thyroglobulin and keratin of mammals.

Partial Antigens or Haptens. It has been noted above that a complete or true antigen is capable of stimulating antibody production and of combining with such antibodies in a specific manner. Certain carbohydrate and lipoid substances, though unable to incite the formation of antibodies when injected alone into the tissues, nevertheless, become fully antigenic when combined with proteins. Further, they can specifically react with the antibodies formed by the combined product. Thus, they display a partial antigenic activity. Such substances are, therefore, called *partial antigens* or *haptens*. It has been established that the specificity of many of the bacterial antigens is dependent on non-protein fractions linked with protein carriers. The best known example is the capsular substance of the pneumococcus, also known as the *specific soluble substance* abbreviated to S.S.S. It is a complex polysaccharide of high molecular weight. By itself it fails to incite antibody formation, but when mixed with the pneumococcal protein it becomes fully antigenic and leads to the production of antipneumococcal substances with which it reacts in vitro specifically. It is clear that the antigenicity in this case depends on the carbohydrate-protein linkage. The exact nature of such linkage is not known. But then, it is the capsular polysaccharide that determines the immunological specificity of the pneumococcal types. When that is lost and the organism becomes a non-encapsulated rough variant, it can no more be typed; with the capsule the type specificity has disappeared. The protein of the pneumococcus is non-specific and common to all the types; alone it can produce antibodies, but these are not type-specific, reacting with the rough variant of any pneumococcal type.

This polysaccharide-protein linkage of the pneumococcus is not an isolated instance; a variety of other bacteria, such as Friedlander's bacillus, meningococci, haemolytic streptococci, staphylococci and others, exhibit a similar association. In all such cases it is the polysaccharide moiety that confers specificity.

Early studies on Forssman antigen led to the belief that lipoids might function as haptens. Subsequent investigations have revealed that Forssman antigen itself is a complex lipopolysaccharide. This discovery has thrown considerable doubt whether lipoid by itself can play the role of a partial antigen or decide the immunological specificity of antigenic proteins. The view has even been expressed that specificity in all cases is determined by a carbohydrate-protein linkage, irrespective of the presence or otherwise of lipoidal substances. Whether the lipoid in the Forssman complex has any share in determining the specificity is a question that can only be decided by further study. The lipoid-carbohydrate combination in the hapten is peculiar to the heterophile antigen of higher animals; so far as our knowledge goes, lipoidal substances do not occur in the heterophile antigen of bacteria.

Numerous investigations have been made to discover whether any non-protein substance, other than protein-linked carbohydrates and lipoids, can act as true antigens. There are several difficulties in the way of the experimental worker. Substances for study should be obtained in an absolutely protein-free state and it is well known that even traces of protein may be sufficient to elicit antigenic response. Again, when such a purified substance is injected into the experimental animal, there is no certainty that it will not combine with some protein of that animal, leading to the formation of an antigen complex and so ultimately to a false result. Evidence so far as it goes tends to show that such a possibility cannot altogether be ruled out. The results of all attempts so far made to solve the above point are inconclusive and for the present we may take it that non-protein substances are not generally antigenic.

Heterophile or Heterogenetic Antigens (Forssman Antigens). An emulsion of guinea-pig's organs when injected into rabbits incites the production of a lytic factor against sheep corpuscles. Extract of the organ with alcohol gives a lipoidal complex containing probably a polysaccharide moiety also. This lipoidal substance will react in vitro with the haemolytic antibody, but is unable to induce the same antibody production when injected alone into the rabbit. When the macerated tissue after extraction is injected no haemolysin is formed. On recombining the macerated tissue with the extract and injecting into rabbits after

allowing the mixture to stand for some time, the same haemolysin is generated. It is, therefore, manifest that the guinea-pig organs contain a complete antigen in which a protein fraction is linked with a lipoid-carbohydrate complex which behaves as a hapten. Because this complex antigen does not follow the species-specific rule of ordinary antigens, it is called *heterophile antigen* or *Forssman antigen* after the first discoverer.

Heterophile antigens are extraordinarily heat stable, withstanding the boiling temperature for several hours. Likewise, they withstand treatment with alcohol or ether for many days with no evidence of deterioration. The antibodies produced against them are called heterophile antibodies. In general, heterophile antibodies can be produced only in such animals as do not have the heterophile antigen in their organs or blood. The heterophile lysins are different from the species-specific haemolysins. It must be evident from the above that sheep cells should contain the heterophile antigen besides the species-specific antigen. If whole sheep cells are inoculated into the rabbit, both types of haemolysins are formed. But if the cells are injected after boiling, only the hetrophile lysin is formed. It may be mentioned that certain of the natural antibodies are of the Forssman type, *e.g.* the haemolysin for sheep's erythrocytes found in normal human serum.

Heterophile antigens are widely distributed among animals in a random fashion. They are found in the organs and tissues of the guinea-pig, horse, dog, cat, mouse, fowl, tortoise, several species of fish and in a few bacteria, such as some of the paratyphoid and dysentery bacilli and pneumococci. The erythrocytes of sheep, but not its organs, contain heterophile antigens. These are absent from the organs of man, rabbits, cattle, pigs, rat, and a number of other animals. It is interesting to note that a Forssman antigen is present in the red cells of Groups A and AB and it is closely related antigenically with the Group A substance which is a polysaccharide. About the group antigens found in man's erythrocytes reference will be made in a later section.

Antigenic Structure. The antigenic structure of the bacterial body is highly complex and bears a close relation to virulence. Several kinds of antigens are found constituting the bacterial body (antigenic mosaic). Although they are all protoplasmic

in nature, they display qualitative differences, conditioned by their chemical make-up. In other words, the chemical structure of any organism, as that of all forms of life, is never simple. In revealing this complex structure of bacteria, no method is so delicate or sensitive as the immunological method. But primarily these antigens are in a great many cases isolated by biochemical methods. In all such methods their solubility or otherwise in solvents like alcohol, trichloracetic acid, glycols and others forms the mainstay in the technique of isolation. There is no clear indication as to the exact position in the cell of these different antigens, except that they are found in some cases superficial, as for instance, the capsular antigen, the labile antigens or the smooth somatic antigen of the *Salmonellas*. The conception that they are found in layers on the bacterial body is not supported by any conclusive proof.

As noted above, the same bacterial species may contain multiple antigens; for example, the typhoid or the Flexner bacillus contains more than one complete antigenic component. Antigens may be those residing in the body, the *somatic* type, or those residing in the appendages, the *flagellar* or *capsular* type. Each of these types may be constituted of more than one kind of antigen. In most bacterial species the antigen situated outermost on the body is a smooth antigen, the smooth somatic antigen, which is also the virulent antigen of the cell. Superficial to the smooth somatic antigen is found in a few cases another somatic antigen called the *labile antigen*. The flagellar antigen, also called the *H antigen*, of a motile organism is immunologically distinct from its somatic or *O antigen*. Again, in a capsulated organism, the antigenic nature of its capsule is different from that of the body substance. Under certain conditions, the somatic antigen may undergo variation, the new antigen thus revealed displaying characteristics quite different from those of the original type (Chapter XIV). Closely related species of bacteria may possess certain antigenic components in common; that is, they share an identical antigen or antigenic fraction. These are referred to as *group antigens*. This explains the group reactions between such bacteria. As mentioned above, widely different groups of organism also may share identical antigenic components, e.g. *Rickettsia* and *Proteus*, which account for the cross reactions observed among them. Some people, however, believe that such cross

reactions are due not to a sharing of antigens but to the occurrence of similar determinant groups.

Antibodies. Antibodies are protective substances formed by the tissues of the host in response to the action of antigens and reacting specifically with them. They appear in the blood in the maximum concentration. When brought into contact with the poisonous antigens which incite their production, they combine with them, not only in vivo but also in vitro, and in a general way tend to counteract their toxic effect.

The serum which contains antibodies is called *immune serum* or *antiserum*. The presence of antibodies in it is demonstrable in vitro by the reactions they give rise to when the immune serum is mixed with the homologous antigen. The antibody that is produced against an extracellular toxin is called *antitoxic antibody*. This type of antibody, as instanced by the diphtheria or tetanus antitoxin, combines specifically with the toxin and neutralises it. The antibodies induced by the action of endotoxins are called *antiendotoxic* or *antibacterial* antibodies. They react with the respective antigens in a variety of ways and their terminology is descriptive of the type of reaction they cause as the result of combination. Thus, the *lysins* cause the dissolution of bacterial or other cells; the *bacteriolysin* causes the lysis of bacterial cells and the *haemolysin* the lysis of red blood cells. The term bacteriocidin is sometimes used when there is simple killing of bacteria without lysis; there is no separate antibody involved in it; it is but one of the properties of lysin. The *bacteriotropine*, like the natural *opsonin*, sensitises bacterial cells preparatory to phagocytosis. The *agglutinin* brings about the immobilisation of bacteria, when motile, and clumping or agglutination, settling them out of suspension. The *precipitin* aggregates the molecules of soluble antigens and causes the formation of a precipitate. Sometimes the term complement-fixing antibody is applied to indicate an antibody which interacts with the antigen in co-operation with *complement* or *alexin*, a natural non-specific antibody-like substance found in the blood; but the former does not seem to be distinct from precipitin. The term *neutralising antibody* is often applied in a special way to certain neutralising substances formed in viral infections; but it should be understood that this term can be used to any antibody that will neutralise the toxin of an infectious agent. Similarly, the term *sensitising antibody*

is often employed to denote the substance concerned in hypersensitive phenomena. As distinct from the immune antibodies, are the *normal* or *natural* antibodies not infrequently found in the serum of apparently uninfected or untreated man and animals; they display similar effects as those of the former.

Site of Antibody Formation. There is no definite knowledge regarding the exact site of antibody formation. It may be the same tissues or organs where serum proteins, especially globulins, are formed. The implication of Ehrlich's hypothesis seems to be that every cell in the body as a living unit is probably a potential antibody factory; while this is at present no more than speculation, there is ample evidence to show that certain tissues are primarily responsible for the synthesis of antibodies. Experimental evidence tends to show that the reticulo-endothelial system is one such tissue that plays a prominent part in the formation of antibodies. This system, as is well known, is also concerned with the clearing of foreign bodies and effete cells from the blood stream. Hence, it is not unreasonable to assume that it takes up the antigen and during the process of disposal manufactures antibodies, being specifically stimulated by the former. Though widely distributed in the body, this system is concentrated in certain particular organs, especially the spleen, liver, bone marrow and lymph glands. The wandering macrophages of the tissues and the blood also belong to this system. There is reason to believe that the lymphoid tissue, in general, may also be concerned in antibody production.

Wherever produced, antibodies are distributed throughout the body. The concentration in the blood is the highest. They are found in the lymph and most of the body fluids; the cerebrospinal fluid is an exception.

Factors Influencing Antibody Production. The response to antibody production is influenced by numerous factors. The conditions governing it in spontaneous infections are necessarily complex and by no means clear. Wide variations in individual responses are observed. For example, all typhoid patients do not show the same agglutinin titre in their blood on the same day of disease. Again, considerably debilitated tissues are unable to respond to any antigenic stimulus. Such factors as the nature of antigen, the dose, number and route of injection and the animal employed are all of importance in artificial immunisation. All

animals do not react equally well; rabbits are more efficient in this respect than guinea-pigs.

The injection of an antigen is followed by no detectable change for some days; too large a dose will cause a marked deterioration of the defence powers of the body and bring about a marked decrease in the concentration of any specific antibodies that might have existed before. Then the titre steadily rises to a high level. This lag period is called the *negative phase*. It is important in that its duration affords an indication regarding the spacing and dosage of injections. After reaching a maximum level, the titre falls, rapidly at first, then slowly and after a time antibodies disappear altogether from the blood. Such an individual shows an immunological state different from that of the normal. He retains a basal immunity as manifested by a ready and marked response to a subsequent injection of the same antigen. In a person from whom the circulating antibodies have thus disappeared, they may reappear, though in a low titre, under the stimulus of a different antigen introduced on a subsequent occasion. It is a non-specific stimulation; but we must remember that antigens are shared by several microbial species and it is not without the possibility of being a specific one. For example, a person who is immunised against *Bact. typhosum* shows at a future date an increase of the antityphoid agglutinins, or their reappearance after complete disappearance, in his blood in consequence of some other infection, say for example influenza. This kind of reaction is called *anamnestic reaction* (anamnesis = recollection). It is important to bear in mind that in the case of flagellated bacteria this kind of non-specific stimulus does not affect the somatic agglutinins; it is the flagellar agglutinin that may be so incited.

It may be noted that the defence mechanism of the body is subject to stimulation by any antigenic substance; a general mobilisation of its resources, in addition to the specific, follows such stimulation. This forms the basis of non-specific protein therapy; the injection of sterile milk or T.A.B. vaccine, for example, heightens the general resistance of the body to infection. Vaccines may owe part of their value to non-specific effects. Hence, we should be cautious in the interpretation of the results of vaccine therapy.

The tissues responsible for the production of antibodies display a characteristic difference in their behaviour towards the

first injection and the following ones. A second injection leads to a more rapid and much more copious production of antibodies than the primary injection. This is also important in determining the number of doses to be given for efficient immunisation. That is why in prophylactic vaccination against enteric fever, cholera and others two doses are preferred to one. The first dose sensitises and primes up the tissues so that response to a subsequent dose is immediate and more energetic. In the latter no negative phase, or preparation period, is needed. It may also explain how second attacks are warded off or rendered mild. Hence, not only in the presence of antibodies, but also in the capacity to generate them spontaneously and abundantly, does an immunised person differ from a normal one.

A simultaneous injection of a number of antigens results in the production of antibodies for all, but the titre of the individual antibodies may vary considerably. Typhoid vaccine, for example, usually contains the typhoid, *paratyphoid A* and *paratyphoid B* bacilli (T.A.B.). Protection against all these is simultaneously developed by its injection.

Nature of Antibodies. Not much is known regarding the exact chemical nature of antibodies. Their chemistry is intimately bound up with the chemistry of serum proteins. Antibodies are believed to be protein in nature. Two views have been expressed regarding their relation with the serum proteins; one is that they are modified serum globulins and the other that they are new substances but closely associated with the serum globulins. Although there is no conclusive proof to show that they are serum globulins which have undergone some specific change under the impact of antigenic stimulus, that is the view now held by many immunologists (see below). Several attempts have been made to demonstrate a quantitative increase of the serum globulins in the immunised subjects, but the results are conflicting. Equally unproved is the assumption that antibodies are independent entities. But there is ample evidence to show that they are intimately associated with the serum globulins. The association of different types of antibodies with particular globulin fractions lacks regularity, some appearing with pseudoglobulins, some with euglobulins and others with both, depending upon the animal immunised and the nature of the antigen. However,

their association with globulin provides means for the concentration and purification of therapeutic sera.

Methods usually employed for precipitating the serum globulins are salting out with ammonium or potassium sulphate, treating with alcohol at various temperatures or dialysis. Isolation may also be effected by enzymic action. By peptic digestion of serum under certain temperature (37° C.) and pH (4·2) conditions, the inert albumin can be almost completely removed, leaving the antibody globulin almost intact. After separation of the globulin, concentration is but a simple step.

The separation of antibody globulins from other globulins can be effected by taking advantage of their specific combination with the corresponding antigens; the resulting antigen-antibody complex is then separated into its constituents by dissociation or selective digestion with trypsin. Cellular antigens are not suitable for this purpose, as they adsorb only a very thin layer of antibodies. But relatively large amounts of antibody are precipitated by soluble antigens, like proteins and the polysaccharide haptens; the latter as they do not contain nitrogen, are particularly suitable for this purpose. By employing the above methods, a diphtheria antitoxin of high purity has been prepared: so also antibodies of certain pneumococcal types. Such purified antibodies, in their elementary composition and general physical and chemical properties, are hardly distinguishable from globulin.

There is another way in which the specific isolation of antibodies has been accomplished. When natural proteins are used as antigens, the antigen-antibody complex is a mixture of two proteins which are very difficult to separate. This difficulty has been overcome by employing antigens which are readily identifiable chemically, like haemoglobin, coloured azoproteins and proteins containing tracer elements, like iodine, phosphorus and others. The antibodies are colourless and so their amount in the precipitate can be estimated without difficulty by chemical analysis.

Estimation of the relative proportion of the ingredients has shown that the bulk of the precipitates is composed of antibodies. In composition, general characters, molecular weight, electrophoretic behaviour and other physicochemical properties, the modified globulin reveals no material difference from the normal serum globulins, except that the former displays relatively greater

resistance to the action of proteolytic enzymes. Thus, all attempts to separate antibodies from globulins and to obtain them in a protein-free state have so far ended only in failure. But from these negative results the old belief that antibodies are modified serum globulins gains greater confirmation. The final proof for or against this belief, however, has yet to be sought for.

By the electrophoretic method the serum proteins can be fractionated with great accuracy. Following this procedure it has been possible to make out three components in the serum globulins; they have been named α, β, γ globulins in the order of decreasing migration speed in the electric field. Antibody appears to be mainly associated with the least mobile γ-globulin and less with the β-globulin; the α-fraction is inactive.

A quantitative increase of the serum globulin, especially the γ-globulins, in the immunised animal has been reported. In general, this increase seems to occur at the expense of albumin, but in some cases it is due to a general increase of serum proteins, which may be up to 15 per cent., with no reduction in albumin. Only less than half of the new globulin displays antibody activity, the rest being serologically inert. Another interesting feature of the antibody globulin is the variability in size of the molecule; this seems to depend, in part at least, upon the animal species immunised. The immune globulin produced in the horse, cow or pig against Type III pneumococcus, for example, has a molecular weight (910,000–930,000) over four times that of the corresponding globulin of the rabbit, man or monkey with molecular weight of 156,000–196,000. This peculiarity is not a feature of all immune globulins; the horse globulins against the diphtheria, tetanus and **welchii** toxins are of the smaller type.

Unity and Diversity of Antibodies. A variety of specific reactions have been mentioned above, such as lysis, precipitation, agglutination, opsonic action and others. Naturally the question arises whether there is a separate and distinct antibody responsible for each reaction. That is what the early observers believed. They regarded that a variety of anitbodies were produced against a single antigen and that the various immunological reactions were fundamentally different from one another. With increasing understanding of the nature of these reactions, however, the idea

began to dawn that the various manifestations of antibody reaction might be due to a single antibody acting in different ways conditioned by the nature of the antigen and the circumstances of action. Thus, even in the early days, the essential similarity between the precipitation and agglutination reactions had been suggested attributing the apparent difference between them to the difference in the physical state of the antigen.

The unitarian hypothesis postulates that there is only one antibody formed against a particular antigen. The antibody combines with the corresponding antigen. By this union the antibody sensitises the antigen and, in so doing, changes its physical and chemical state in such a way that it becomes equally susceptible to any of the actions—lytic, opsonic, agglutinating, precipitating or complement-fixing. What particular action is to follow sensitisation is determined by the physical state of the antigen, whether cellular or particulate, and also by the particular circumstances in which the union of antigen and antibody takes place, such as the presence or absence of complement, leucocytes and so on. That is to say, these differences in reactions are not due to any differences in the nature of the antibody but to the differences in the circumstances of reaction.

The unitarian conception does not imply that a complex antigenic source consisting of multiple antigens produces only one antibody. For example, a bacterial cell, like the typhoid bacillus, is such a composite antigenic entity, each component of which is capable of independent antibody stimulation. Consistent with this knowledge the unitarian school recognises that in response to the introduction of such a complex antigen into the tissues, separate antibodies will be produced against each of the antigens constituting the complex source, though these multiple antibodies may have different titres. But this does not weaken the unitarian hypothesis which only lays down that each antigen produces only one antibody.

Proof for the unitarian hypothesis has been sought for in a variety of ways and a large body of experimental evidence has been brought forward in support of it; for instance, the occurrence of agglutination of erythrocytes with a specific serum when complement is absent and of haemolysis when it is present, agglutination or phagocytosis of antigen-coated collodion particles by precipitating sera, fixation of complement by the precipitate

after precipitation reaction is over and others. These observations tend to confirm the essential unity of serological reactions and the present trend, is, therefore, towards an acceptance of this theory.

Mechanism of Antibody Formation. Our knowledge about the exact mode of formation of antibodies is very meagre. An early suggestion was that the antigen itself was in some way transformed in the tissues into antibodies. But the evidence against it is so overwhelming that it need not now be seriously considered. Another interesting and comprehensive attempt to explain the origin of antibodies and the mechanism of serological phenomena was the *side chain theory* formulated by Ehrlich. It was highly speculative and its main value lay in the fact that it gave a tremendous impetus to the study of immunological problems in general.

The cell according to Ehrilich consists chiefly of a central chemical nucleus deriving its nutriment from the blood or lymph, in which it is bathed, through the agency of certain atom groups called *receptors* or *side chains* located on its surface. The receptors are conceived to have the same relation to the cell nucleus that the side chains of complex molecules bear to the central nucleus of the molecule. From this is derived the name side chain theory. The side chains may be simple in structure adapted to fix simple food molecules or more complex and able to take up highly complex protein molecules. Each cell has on it innumerable receptors of different structure and capacity. They normally function as points of attachment for nutrient particles which are hooked and altered before incorporation. Under abnormal conditions such as occur when antigens, bacteria, their toxins, foreign cells or other substances, are introduced into the body, the side chains display the same activity towards them as for food particles. Antigen, in their turn, can exert their activity only if they are attached to these receptors. In dealing with foreign substances, the antigens, the side chains are diverted from their normal function of assimilation and may be damaged or destroyed. If the resulting damage to the cell is not considerable, active repair takes place. This injury provides a powerful stimulus for the production of new receptors of the same type as those thrown out of gear and destroyed. Overproduction follows in accordance with the usual physiological habit. Due

to the consequent overcrowding, the excess receptors are shed into the surrounding tissue fluids and passed into circulation. Such free receptors form specific antibodies. They vary widely in their structure and chemical affinities.

Though separated from the cell body, the receptors still retain the power of combining with the homologous antigen, *i.e.*, they can unite with the antigen specifically; the nature of this union is chemical. In order to explain the various types of antigen-antibody reactions, Ehrlich postulated the existence of three types of receptors, each provided with anchoring or *haptophore* groups. He further postulated that antigens are also similar in structure, containing both haptophore and toxophore groups. The first and simplest order of receptors has only one haptophore group: it simply combines with the antigen and neutralises it.

Antitoxin belongs to this order. Toxin is regarded as a relatively simple substance possessing a haptophore and a toxophore group. Union takes place between the haptophore group

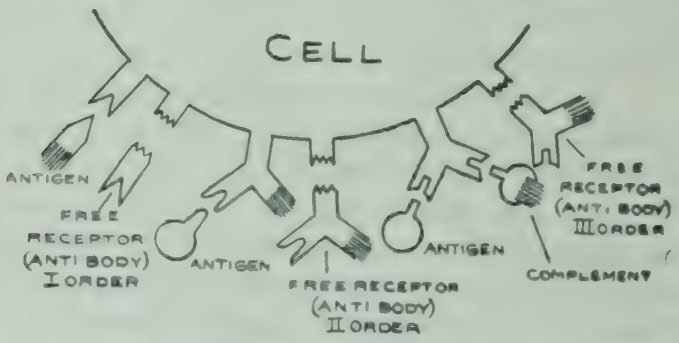


FIG. 12. **Schematic Representation of Ehrlich's Side Chain Theory.**

of the receptor and that of the toxin which is thus prevented from anchoring itself on to the intact receptors on the cell and damaging it. The result of the union is, therefore, a simple neutralisation of the toxin. The toxoid according to this conception is regarded as an inert form of toxin in which the toxophore group is destroyed, while the binding or haptophore group remains functional. The second order has a single haptophore group and a *zymophore group*. The latter determines the kind of change, precipitating or agglutinating, that should befall the antigen after fixation. The third order has two haptophore groups, one to fix the antigen and the other to fix the complement

(alexin) which is also regarded as possessing a combining and a determinant group. The former of the two is called the *cytophile group* and the latter the *complementophile group.* As there are two receptor mechanisms of the haptophore type in this order, the term *amboceptor* is applied to it. The cytophile group sensitises the cell and prepares it for the action of the complement that is likewise fixed. All antibodies, that require the intervention of complement for their action, belong to the third order.

Thus, according to Ehrlich antibodies are free receptors broken off from the parent cell and released into circulation. They combine with the antigen in one way or other, as indicated above, and give rise to the various immunological reactions. Although many of his postulates have been confirmed by recent investigations, there are others which are irreconcilable with observed facts. His premises themselves are uncertain and require proof. For instance, the structure of the cell as conceived by him is untenable in the light of modern knowledge and proof is lacking that antibody production is analogous to the process of assimilation. It will be noted that in spite of the enormous work done subsequent to Ehrlich's hypothesis, the mechanism of antibody production still remains largely unsolved. Mostly on circumstantial evidence, we assume that antibodies are synthesised in some manner by the tissues under the stimulus of antigen. If the view that antibodies are modified serum globulins is accepted, then the mechanism of globulin production and its modification is also the mechanism of antibody formation.

Regarding how the serum proteins are produced our information is scanty; nor do we know the exact cell groups which are responsible for their production. The large antigen molecules have on their surfaces a number of atom groups which are characterised by certain configuration, spacing and electrical force. When we say that an antigen stimulates the production of antibodies, what we actually mean, if we accept the modern view regarding the nature of antibodies, is that the antigen interferes with the normal process of globulin synthesis, leading to the formation of abnormal globulins. No differences in the physical and chemical properties, it may be stated, have been established between the abnormal and normal globulins. During production the globulins are made to adapt themselves to the determinant groups of the antigen molecules so that the finished

globulin molecules will have their pattern complementary to that on the antigen molecules. Clearly then, the nature of the surface pattern of any antibody globulin will depend upon the surface pattern of the particular antigen employed in its production and the globulin adapted to one antigen will be a misfit for another antigen. How exactly this adaptation takes place is now a matter of speculation. It has been suggested that the synthesis of immune globulins takes place in the presence of antigen within the synthesising cell. The antigen present provides the pattern on which the normal globulin molecules are moulded with the result that the structure of the finished product, the modified or immune globulin, is complementary to the antigenic surface. Dissociation of the newly formed antibody globulin from the antigen molecule then takes place, freeing the latter, and the process is repeated with the same antigen molecule as the pattern.

The structural adaptation of the antibody to the polar groups of the antigen molecule may not be thorough and the new globulin molecules may not show uniformity in the degree of adaptation. A monospecific serum, *i.e.* a serum prepared against a single antigen, may contain both perfectly and imperfectly adjusted antibody molecules. A perfectly adjusted antibody will form a firm union, whereas an imperfectly adjusted one will form a loose union which is easily reversible. In the latter case no visible precipitation will follow the specific union. It should be remembered that the whole hypothesis rests on the belief that antibodies are modified globulins, a proposition that still awaits final proof.

Valency of Antigen and Antibody. Of late, evidence has been accumulating to show that both antigen and antibody are heterogeneous with respect to their reaction capacity. That is to say, the molecules of any antigen or antibody do not have the same uniform reactive avidity; some are actively reacting, while others are feebly reacting. Mention has already been made that the present conception of antigen-antibody reaction is that the first stage of the reaction, *i.e.* specific union, takes place in virtue of certain active groupings present on the constituent molecules of the antigen and antibody. It has been suggested that the observed fact of heterogeneity may be ascribed to a difference in the number of such active groupings on individual

molecules. Such reactive groupings are sometimes spoken of as *valencies*. They are not simple atomic valencies as in chemistry but represent atom groups with a definite spatial arrangement on the molecular surface and endowed with electrical force which may vary in intensity.

There is sufficient evidence to show that antigens are multivalent. That is to say, they possess a number of combining complexes on their molecular surfaces. Analysis of the specific precipitate makes it possible to estimate the valencies of antigens. This, of course, gives only the minimum values, for, when there is overcrowding, it is not possible to determine all the valencies because of spatial considerations. The number of valencies varies with each antigen; it is also conditioned on the extent of the molecular surface. The ovalbumin molecule, for instance, with a molecular weight of about 42,000, shows a functional valency of about 5, whereas the thyroglobulin molecule, with a molecular weight of 650,000, shows 30–40. Artificial antigens may show much higher valencies.

Many workers now believe that antibodies are divalent. This conception offers a simple explanation for some of the discrepancies in the behaviour of antibodies. Even a monospecific serum displays lack of homogeneity in its reactive capacity as an antibody, some of the molecules combining with the antigen with great avidity and others with feeble avidity. This variability has been explained on the basis of a difference in the valencies of the molecules of antibody globulin, the least active molecule being monovalent and the more active ones divalent. On the basis of this conception, it is not difficult to conceive that when specific union, or the first stage, takes place but without proceeding to the second stage, the reacting antibody is monovalent; conversely, when the antibody molecule has more than one valency, the reaction will proceed to completion. It is also possible that loose union and suppression of flocculation in a precipitating system may be due to imperfectly adapted polar groups (see above).

Nature of Serum Reactions. As seen above, Ehrlich's side chain theory also seeks to explain the mechanism of antigen-antibody reactions. According to him the shed cell receptors, which constitute antibodies, are chemical entities; they combine with antigens and neutralise their effect in a variety of ways; this neutralisation is similar in character to that of a strong

acid by a strong base. In other words, the union of antigen and antibody is a firm one and takes place in constant proportions. Some of these conceptions are untenable in the light of many observed facts of serological reactions, particularly of toxin-antitoxin reaction. In certain circumstances dissociation of the antigen-antibody complex is possible, which militates against the firm union hypothesis: similarly with the constant proportion concept as shown by the observations of Danysz, and other phenomena. But it should be noted that the chemical specificity of antigen-antibody reactions, an outstanding contribution to the study of the mechanism of these reactions, is a main point in Ehrlich's side chain theory.

Bordet refuted Ehrlich's theory of chemical union. He regarded the antigen-antibody reaction as more physical in nature than chemical and sought to explain most of the phenomena connected with the serum reactions on the basis of adsorption, thus placing them in the category of colloidal reactions. The antigen and antibody solutions display in many respects characters of colloidal systems. During their union the antibody molecules are regarded as being adsorbed on the surface of the antigen molecules in a thin film. By this process the antigen, cellular or particulate, is sensitised when it is readily agglutinated by salt solution, thus explaining the precipitation and agglutination phenomena. Sensitisation also endows the antigen with complement-fixing capacity which may or may not lead to lysis, conditioned by the nature of the antigen. According to this theory the quantitative relationship between the interacting antigen and antibody could be reconciled with the observed fact, namely that the interaction takes place in varying proportions instead of in constant proportions, as union between two colloids may take place in varying proportions. Phenomena like that of Danysz thus found a satisfactory explanation. But he was not able to explain the principle of specificity on the basis of his theory. Why antibodies against *V. choleræ*, for instance, are adsorbed only on cholera bacilli and not on dysentery or typhoid bacilli does not find an explanation in this theory.

Arrhenius and Madsen, while agreeing with the chemical nature and specificity of the reactions as propounded by Ehrlich, however, differ from the latter in certain respects. They consider that the reaction does not resemble that between a strong base

and a strong acid but is analogous to the reversible action between weak bases and weak acids, in which the equilibrium of reaction depends on the concentration of the interacting substances in accordance with the law of Mass Action. This theory also does not fit in with all the observed facts.

None of the above theories can alone explain satisfactorily all the phenomena involved in immunity reactions. The present conception of the mechanism of antigen-antibody reaction is a selective combination of the teachings from all these theories. Into the main teaching of the chemical specificity of Ehrlich, largely extended by the studies of Landsteiner and his colleagues, is incorporated the varying proportion concept of Bordet. It also accepts with modification the view advanced by Arrhenius and Madsen that the antigen-antibody complex may be dissociable.

As mentioned before, the antigen and antibody molecules have a complementary surface structure. The determinant groups on their molecular surfaces are electrically charged, each group being surrounded by a narrow field of force. The actual union of antigen and antibody molecules is due to this electrical force or polar force. But an essential condition of the antigen-antibody union is the complementary nature of the molecular shape. For, without this, close contact between molecules will be impossible for sterical reasons. The electrical force of each atomic group acts only within a very narrow radius: hence these forces cannot be effective unless they come very close together. Since the shape of the antibody molecule is adapted spatially to the superficially placed polar groups of the antigen molecule, larger areas of each molecule are able to come close together with the result that a larger number of force points come into apposition and meet. Thus, the total attractive force becomes so strong as to bind together the molecules of the antigen and antibody. In so doing the small water molecules that hydrate the polar groups of the antigen molecules are probably pushed out by the specifically modified polar groups of the antibody molecules. This union of antigen and antibody molecules constitutes the first stage of all serological reactions. It is followed by various secondary phenomena which have been dealt with in another place.

Natural Antibodies. The normal sera of man and many animals frequently contain various kinds of antibodies. For example, the sera of cattle are often seen to contain antibodies

against the yellow fever virus and agglutinins for organisms such as the cholera, typhoid, dysentery and certain *Salmonella* bacilli; not infrequently again diphtheria antitoxin is found in the human and horse sera; similarly, agglutinins, indistinguishable from the immune agglutinins, are found in the human sera against the chloera, dysentery, enteric, *Brucella* and other organisms, for which no apparent cause can be traced. The titre of these natural antibodies is generally low. Their origin is obscure, but it is likely that they arise in more than one way. The well known transplacental source cannot account for it, for the maternal antibodies so transmitted tend to disappear within the first year of life. One view is that, in some cases at least, the natural antibodies are produced as the result of environmental stimuli; that is to say, repeated inapparent natural infections cause their production. Thus, it is highly probable that the so-called normal antitoxins of diphtheria and agglutinins against the typhoid, cholera and other organisms are really immune antibodies generated as the result of contact with the specific bacteria through subclinical infection. However formed, they may be taken as reinforcing the defence power of the body. Whenever such natural antibodies are present, the body is found to react more vigorously to specific stimuli, natural or artificial.

The genesis of some of the natural antibodies, however, still remains a mystery. It cannot be explained on grounds of subclinical infection as in those instances, in which antibodies may be demonstrated, no probability of specific contact with the antigen can be established. An explanation has been offered on the basis of common antigens; according to this, individuals who show the presence of such antibodies must have come in contact with apparently unrelated bacteria, not necessarily pathogenic, which share immunologically the same antigen. Such random distribution of immunologically identical antigens has been demonstrated in many instances: *Rickettsia* and the X strains of *Proteus*; capsular antigen of Type II pneumococcus and Type B Friedlander's bacillus, certain species of yeast, certain vegetable gums like gum acacia and some strains of *Bact. coli* and *Bact. aerogenes;* the plague bacillus and *paratyphoid B*; the polysaccharide of Type XIV pneumococcus and the specific Group A antigen of human blood. Possibly, therefore,

contact with such chemically, but not biologically, specific antigens may explain the origin of some of the natural antibodies.

Certain types of antibodies, such as haemoagglutinins which determine the human blood groups, are produced in the absence of antigenic stimulus. They are genetically developed and form part of the physiological make-up of the body. Again, a few of the natural antibodies are of the heterophile type; such is the case with the natural haemolysin for sheep's erythrocytes, that is found in the human and rabbit sera. In this connection, it is interesting to note that a haemoagglutinin for sheep cells develops (Paul and Bunnell) in the blood of patients suffering from glandular fever.

Antitoxic Immunity. Our present knowledge about antitoxic immunity is based on the work of Behring and Kitasato (1890). They found that repeated injection of rabbits and mice with sublethal doses of tetanus toxin protected them against this toxin. Moreover, the serum of such an animal was found to be capable of conferring immunity on non-immunised animals. Further, such sera were found to be able to specifically neutralise tetanus toxin. From these observations they came to the conclusion that the protecting or neutralising effect of the serum was due to the presence of a substance produced de novo by the tissues under the stimulus of the toxin and discharged into the blood. This substance they called *antitoxin*. Subsequent work showed that the range of antitoxin production in not confined to the tetatnus toxin but extended to all other extracellular toxins and also to certain plant and animal poisons, like ricin, abrin, snake venom and others.

Immunity to extracellular toxins thus depends upon the presence in the circulating blood of antitoxins which possess the property of specifically uniting with and neutralising the toxin. The toxin on entry into the circulation is bound by the antitoxin and rendered innocuous before reaching the susceptible tissues. But it is not destroyed by the union. Neutralisation of toxin by antitoxin occurs also in vitro. The ability of the antitoxin to neutralise toxin is demonstrated by what is known as the neutralisation or protection test. Harmful results follow the inoculation of a suitable dose of toxin into a susceptible animal, such as a guinea-pig or mouse, but when appropriate amounts of the toxin and homologous antitoxin are mixed in vitro,

allowed to stand for a definite time interval and injected into the test animal, no injurious effect is produced, proving thereby that the antitoxin has neutralised the toxin.

The union of antigen and antibody is a loose one, not causing the total destruction of either. By appropriate means the combination can be split up. The simple dilution of a neutral mixture of toxin and antitoxin, heating where the antitoxin is relatively less resistant to heat, treatment with hydrochloric acid and freezing in the presence of an antiseptic, like phenol or tricresol, may all cause greater or less dissociation. Certain factors, such as temperature, concentration of the interacting substances and others, influence the rate of reaction between toxin and antitoxin. Another influencing factor, apart from potency, is the *avidity* of the antitoxin for its toxin. This quality is a variable factor; for instance, the diphtheria antitoxin displays greater avidity for its toxin than the tetanus toxin has for its own. Avidity expresses itself by rapidity of flocculation; the more avid an antitoxic serum, the greater the readiness with which it flocculates on mixing with its toxin. Some workers consider that firmness of union between toxin and antitoxin is the more important content of the quality of avidity than promptness of action. Whatever that be, sera which possess this quality appear to afford better protection than slowly flocculating ones. On what exactly does this property depend is not clear; probably the type of the serum protein with which the antitoxin is associated is a determining factor. Another important consideration to be taken note of in the quantitative determination of toxin and antitoxin is the presence, and also concentration, of toxoid with the toxin under test. When it is recalled that toxoid, though devoid of toxicity, still retains its combining capacity, the importance of it in neutralisation experiments will become self-evident. And yet another factor that affects the union between toxin and antitoxin is the manner in which the mixing of the two is effected—*Danysz phenomenon*. Clearly then, the neutralisation of a toxin by the corresponding antitoxin is by no means a simple process; quite apart from the factor of the relative quantities of the reagents, several physicochemical influences enter into the picture.

Danysz Phenomenon. Danysz observed that the amount of toxin neutralised by its antitoxin varies according to the way in which the addition is made. A greater amount of toxin is

neutralised if the toxin is added to the antitoxin all at once than when the same quantity is added in several fractions at relatively long intervals. For example, if x units of toxin are added at the same time to an equivalent amount of antitoxin, the mixture is non-toxic; if, on the other hand, the same amount of toxin is added in three or four fractions at intervals of say fifteen minutes between each addition, the mixture will be highly toxic, indicating that some of the toxin is left uncombined. The explanation of this effect appears to be that the toxin is capable of uniting with the antitoxin in multiple proportions. Thus, the earlier fraction must be combining with more than its equivalent amount of antitoxin, leaving insufficient free antitoxin to neutralise subsequent fractions.

A feature of antitoxic immunity is that a relatively low concentration of antitoxin in the blood is sufficient for protection against natural infections. About 0·01 unit of antitoxin per cubic centimetre of blood would seem to be enough in diphtheria to render a person immune. In such a person as quickly as toxin is liberated from a focus and absorbed into circulation, it is taken up and neutralised by the antitoxin. At the same time, further immunisation of the tissues also progresses side by side. It is important that neutralisation of toxin should take place before it reaches the susceptible cells. Once the toxin has reached the cells, antitoxin is relatively ineffective and very much larger amounts will have to be employed to produce any effect. For example, when once tetanus toxin has gained entry into the motor neurons and symptoms have set in, antitoxin has been found practically impotent to counteract the toxic effect. Hence the supreme need for administering antitoxic sera as early in the disease as possible.

Antitoxins are actively formed by the tissues as the result of stimulation by toxins and are found in the maximum concentration in the blood. Active formation occurs in natural infections or as a result of inoculation with toxin or toxoid, as in diphtheria prophylaxis. The latter method also finds application in the immunisation of animals for the production of prophylactic sera. By the removal of serum from an immunised person or animal, the antibodies contained in it are not destroyed. Such an immune serum still retains its protective properties and the contained antibodies combine both in vivo and in vitro with the

corresponding toxin and neutralise it. It is, therefore, employed for conferring passive immunity.

Preparation of Antitoxic Sera. Immune sera for therapeutic purposes are usually prepared from the horse. The blood of this animal frequently shows the presence of residual antibodies to many bacterial pathogens. Those infections for which sera are required either affect horses naturally or are capable of easy transfer to them. Besides, horses give a large yield of blood. It is for these reasons that horses are chosen. Selection of animal for the purpose is important; age, colour, food, health, etc., matter a great deal. They have to be carefully looked after, housed in stables with adequate ventilation and properly excercised. For injection, toxoid and later untreated toxins are employed in graded doses at suitable intervals. The course of immunisation extends for long periods. Towards the end of it, test bleedings are conducted to find out the concentration of the antitoxic content. When it has reached a certain high value, the horse is bled by puncturing the jugular vein and the blood is received into large sterile bottles. After separation from clot, the serum is removed to sterile containers and a 0·5 per cent. phenol or 0·3 per cent. tricresol is added to it as a preservative. It is standardised and put in suitable quantities into ampoules which are labelled, packed and sent out for distribution.

Standardisation of Immune Sera. The purpose of standardisation is to assess the protective value of the immune serum in order to regulate the dose. It is done by biological methods in which titration experiments are carried out in vivo by the employment of suitable animals. An arbitrary unit, one for each antitoxin, has been adopted for comparison. This unit, in the case of the diphtheria antitoxin, was originally defined by Ehrlich as the smallest amount of antitoxin that will neutralise 100 M.L.D. of toxin. The minimum lethal dose, M.L.D., which was Ehrlich's unit of toxin, is the smallest amount which when inoculated subcutaneously into a standard test animal will kill it in a certain specified time. The test animal is the guinea-pig and the standard is 250 grams weight. As mentioned previously, toxin deteriorates progressively on keeping or under the influence of a variety of physical and chemical agencies and loses much of its toxicity while still retaining its capacity to combine with antitoxin. There is thus no regular correspondence between

toxicity and capacity for union, except probably with the very fresh toxin. Because of this instability, toxin cannot be preserved as a permanent standard. Hence, M.L.D. as a fundamental unit no more forms the basis of standardisation. Antitoxin is much more stable; when dried in vacuo and preserved at 0° C., it keeps unimparied for a long time and so provides a stable standard of reference. Standards for all antitoxins prepared on similar lines, are preserved in central institutes and can be obtained from there for standardisation of new preparations.

Since standardisation is based on the neutralisation test, it is obvious that every time a newly prepared antiserum is to be standardised a fresh toxic filtrate has to be previously prepared and standardised with reference to the particular international unit of antitoxin. The antitoxic serum under test is then titrated against the newly evaluated toxin. As titrations are performed within a short time after standardisation, there is little risk of the toxin losing its toxicity within that time. Different units of toxin are now employed in titration: the L+ dose (Limes Tod–threshold, death) is the smallest amount of toxin which, when mixed with one unit of antitoxin and injected subcutaneously into the standard animal, will kill it in a specific time. Another unit that is sometimes employed is the Lo (Limes Nul) unit which is the largest amount of toxin which, when mixed with one unit of antitoxin and injected subcutaneously into the test animal, will give rise to no observable reaction. The Lo dose will just neutralise one antitoxin unit. Varying quantities of the serum under test are mixed with the same known quantity of toxin, L+ dose, and the mixtures are individually injected each into a batch of experimental animals and the effect observed. The amount of antitoxic serum, that will neutralise this amount of toxin, as shown by the results on these animals, contains one international unit of antitoxin and from this the number of units contained in a certain volume, per ml., can be estimated. Another method of titration is by the intracutaneous injection of a mixture of toxin and antitoxin into the test animal. It is based on the Lr dose of toxin which is the amount of toxin which, when mixed with a standard unit of antitoxin and injected intracutaneously into the test animal, will produce the minimal skin reaction. The neutrality point can also be roughly estimated by the flocculation reaction of Ramon; when the ingredients

are brought together in different proportions, flocculation occurs most heavily and rapidly at this point, this is the Lf dose of toxin. But this method is less reliable and is only employed as a rough preliminary to the in vivo tests. Details about standardisation are given in the chapter on *C. diphtheriæ*.

The L+ dose is the minimum amount that will cause death of the test animal under certain specified conditions and Lo dose is the amount that will just neutralise one antitoxin unit. The Lf dose is, therefore, larger than the Lo dose and one would suppose that the difference between these two doses will be one M.L.D. This supposition is, however, wrong and the actual difference has been found to be very much higher, varying between 10 and 100 M.L.D. On the other hand, the Lr dose is very nearly equal to the Lo dose, as the amount of toxin necessary to elicit minimal skin reaction is very small. The Lf dose is really a measure of the combining capacity rather than of toxicity, so that the toxoid degeneration, invariably occurring in all toxic filtrates, does not tell upon the dose. Hence, in general, the Lf dose is slightly smaller than any of the other doses. In the administration of sera we are now guided solely by their potency as measured in terms of the above standards. But potency alone does not always depict the full protective value of a serum; its quality (avidity) is also an important factor. But of this we have now no standard.

Concentration of Antiserum. The antibodies are specialised globulins or separate entities intimately mixed with globulins. The globulin can be precipitated out of solution by 50 per cent. saturation of serum with ammonium or sodium sulphate. The precipitate is removed by filtration and dialysed in water to get rid of the salts. It is then resuspended in physiological saline of much smaller volume than that of the original serum. By this process the bulk of the immunologically inert proteins, which cause serum reaction, is also got rid off. The resulting product is, therefore, both concentrated and refined (p. 242).

Mechanism of Antibacterial Immunity. When there is bacterial invasion, the tissues respond by producing specific antibacterial substances which are found in the blood in large concentrations. These antibodies in some way tend to counteract the damaging effect of the bacterial toxins and thus protect the body. As has been pointed out before, the tissues can also be induced to

generate such antibodies by vaccination. Sera containing these antibodies are called antibacterial sera. Other factors which are also vitally concerned with this type of immunity, are certain non-specific agents such as the phagocytes, opsonins and complement. There is ample evidence to show that antibacterial immunity is partly humoral and partly cellular. The former is specific and the latter non-specific. Passive protection is likewise possible with antibacterial sera. These have led to the inference that the humoral part is more important than the cellular; this still awaits elucidation.

The antibacterial immunity is more variable than antitoxic immunity. It is also less effective than the latter. Standardisation is difficult and often the dose is expressed in volumes. Some of the most important antibacterial sera are the pneumococcal, meningococcal, anthrax and plague antisera.

As indicated in an earlier section (p. 238), the antibacterial antibodies function in a variety of ways and their action can be demonstrated in vitro. The precise mode of action is probably to sensitise the specific antigens, leading to their phagocytosis, lysis, agglutination or destructive changes. These reactions have been exploited to form the bases of important diagnostic procedures in clinical medicine. While the bactericidal, lytic and opsonic functions are potent means of resistance, it is not certain whether the in vitro reactions of agglutination, precipitation and complement-fixation occur also in the body. Some of these reactions, by aggregating the antigens in vivo may have a localising effect which is materially helpful in resistance.

CHAPTER XII

IMMUNITY REACTIONS

Precipitation. The principle involved in the precipitin reaction is that when an antigen in solution is brought into contact with its antibody under suitable conditions, specific combination takes place, followed by visible flocculation of the antigen-antibody compound. The reaction is thus a relatively simple one and so this and the allied agglutination reaction are considered before other more complex reactions. Also because of the simplicity of technique, the precipitin reaction, of all the serological reactions, has been the most studied; the results of these studies have shed considerable light on the quantitative aspect of antigen-antibody reactions in general.

Kraus (1897) was the first to describe the phenomenon of precipitation. Working with cholera vibrios and plague bacilli, he observed that when the cell-free filtrates of their broth cultures were added to the homologous antiserum, a visible precipitate was formed. The filtrate contained the soluble bacterial proteins. He also found that the reaction was specific; the cholera antiserum, for example, caused no precipitation with the plague extract and *vice versa*. He called the phenomenon *precipitation* and the concerned antibody *precipitin*. Sometimes the term *precipitinogen* is applied to the antigen. Subsequently, Kraus showed that the phenomenon of precipitation was a general feature applicable to other bacteria as well. Following this, the discovery was made that all complex proteins possessing antigenic properties, such as animal sera, egg albumin, milk and others, behaved in this respect like bacterial proteins.

As mentioned above, it is a simple reaction involving only two immunological reagents—antigen and antibody. No complement is necessary, but the presence of electrolytes is essential. Specific precipitation occurs when a precipitating serum is mixed with a solution of the corresponding antigen in the presence of electrolytes. Certain experimental conditions influence profoundly

the dynamics of the precipitin reaction. The effect of concentration of the reactants is considered below. The rate of interaction between the reagents, and of visible particulation, is quicker in a smaller than in a larger total volume of fluid in the interacting system. The advantage of reducing the volume of the reagents is, therefore, obvious. An increasing concentration of electrolytes up to a point also promotes the speed of precipitation; any increase beyond this, however, has an opposite effect. Temperature also influences in a similar way, hastening reaction with increase of temperature up to the optimum. Temperatures above 37° C. retard the action and may in some cases cause an already formed precipitate to redissolve. For this reason, the test is usually performed at a temperature at or below this level.

Unlike in the agglutination test, the procedure in the precipitation test is to mix the precipitating serum, undiluted or slightly diluted, with falling concentrations of the antigen. It is done in one of two ways. The dilute antigen is kept in narrow test tubes and the serum is slowly layered on the surface; a precipitate appears at the interface as a white or opalescent band—the ring test. Or the reagents are mixed in the usual way when the antigen-antibody complex settles down as a precipitate. The titre of the precipitin content of a serum is expressed in terms of the highest antigen dilution which gives a visible precipitate with the serum. Sera of very high potency, causing precipitation in dilutions of 1 : 1,000,000 to 1 : 5,000,000 may be obtained. This along with the specificity of the reaction renders the precipitin reaction a very delicate test of great practical utility. The end point of the test, performed as above, does not, however, represent the optimal precipitation which is obtained when antigen and antibody are mixed in a certain definite ratio called the *optimal* or *equivalent proportion.* When titration is conducted with varying proportions of antigen and antibody, the mixture in the tube in which precipitation occurs first and most copiously gives the optimum proportion. Two optimal ratios may be defined: the *constant-antigen optimal ratio* and the *constant-antibody optimal ratio.* In the former constant amounts of antigen are mixed with falling amounts of antibody and in the latter constant amounts of antibody are added to decreasing amounts of antigen. The two ratios are not necessarily identical. While

they differ very little in Ramon's diphtheria toxin-antitoxin titrations, in other cases the quantity of antibody required for optimal flocculation when the antigen is kept constant is found to be six to eight times the amount necessary when the antibody is kept constant. The cause of this discrepancy is to be found in the varying proportions of antigen and antibody that go to make up the precipitate and this, in turn, depends upon the relative proportions of the interacting substances.

The concentration of the antibody in the interacting system exercises a much greater influence in the precipitation than in the agglutination reaction. Any increase in the dilution of antibody beyond a certain low limit will interfere materially with the power to form precipitate. The intimate mechanism of reaction is probably an adsorption of antibody by the antigen. Compared to the coarse bacterial cells, the molecules of the soluble antigens are very fine and so the total surface area to be covered by the antibody globulin is very much greater in the case of the latter type of antigens than in the former. Hence, very much larger amounts of antibody globulins are needed to bring about precipitation than to cause clumping of bacteria. This probably explains why dilution of antibody, which causes a fall in its relative proportion to antigen, results in the partial or complete suppression of precipitation. In contrast to the precipitation reaction, a positive agglutination reaction can still be obtained even after considerable dilution of the specific serum. The above considerations make it manifest that the ratio of antibody to antigen is greater in the precipitate than in the agglutinated bacteria.

In titration experiments following the constant-antibody method, there is a partial or complete inhibition of reaction in the zone of antigen excess. From the above quantitative considerations it is easy to visualise that in circumstances of moderate antigen excess the amount of antibody globulin available is inadequate to fully sensitise all the antigen molecules; consequently, only partial reaction takes place in this region. When the antigen excess is marked, the inadequacy is so gross that no union of antigen and antibody ensues and the result is a complete inhibition of reaction.

Quantitative analysis has shown that in most cases the precipitate is largely formed by the antibody. Even in the same

precipitating system, the amount of precipitin in the precipitate has been found to vary with the concentration of the reactants. These experiments suggest that the antigen-antibody union occurs in varying proportions.

The present conception of the mechanism of the precipitin reaction is somewhat as follows: The reaction occurs in two stages. First there is a specific union between the antigen and the antibody, resulting in the formation of an antigen-antibody complex without visible flocculation. It is a process of sensitisation. It is followed by the second phase in which aggregation of the fine sensitised particles into macroscopic size occurs; this is non-specific and depends on the presence of electrolytes. Both bacterial proteins and antibody globulins are in fine colloidal solutions. They are hydrophilic colloids composed of molecules having certain surface groupings which display great affinity for water. These molecules, therefore, attract a film of water around them, thus maintaining themselves in watery solutions. The specific antigen-antibody combination occurs by the antibody globulin forming a coating around the antigen molecule and in this process the globulin molecules become denatured and transformed from the hydrophilic into the hydrophobic type; the hydrophilic groupings are put out of action partially or completely. When so denatured, a protein colloid becomes salt sensitive. The attraction between its own molecules becomes relatively greater but still not sufficiently great to cause visible particulation. The molecules of the reagents are held in solution not only due to the hydration but also by the like electrical charge each bears. The function of the electrolytes is to reduce the electric charge of the complex, thus reducing or removing the repelling force of the particles which are, therefore, clumped together to form visible precipitate.

An alternate mechanism was propounded by Marrack and is known as the *lattice hypothesis*. Regarding the manner of specific union between antigen and antibody, there is substantial agreement between Bordet's adsorption theory and the lattice hypothesis. Both postulate that the specific union is determined by certain reactive forces present on the molecular surface of the antigen as well as antibody. But the latter theory further assumes that several such reactive bonds are found on the surfaces of the antibody molecules; that is, not only the antigen but also

the antibody is multivalent. And during interaction these mutually fitting force points or polar groups on the respective molocules are brought into apposition when the molecules unite (p. 248).

According to this theory the precipitate in the precipitin reaction is composed of alternate antigen and antibody molecules held together in a mesh-like arrangement, or lattice, by the reactive forces; the antibody molecules are packed around the antigen molecules instead of forming a film. For instance, a divalent antibody molecule can hold two multivalent antigen molecules; these linked antigen molecules combine with other antibody molecules which, in turn, take up fresh antigen molecules and so on till large aggregates are formed. Once started, the reaction is progressive until precipitate is formed. Thus, the actual flocculation is only a natural sequel of the continuation of the initial process of specific union and does not constitute a separate or non-specific phenomenon. This theory, therefore, does not recognise two stages as laid down in Bordet's conception. The hydrophobe transformation of the resulting complex, according to this theory, is due not only to a loss of affinity for water but also to a newly acquired special attraction between sensitised molecules. That electrolytes are essential for the precipitin reaction is not in disharmony with this theory.

Though the precipitin reaction is highly specific, cross reactions with precipitating antisera occur not infrequently. For example, an antihuman serum produced in the rabbit will precipitate sera of chimpanzees, gorillas and higher monkeys. This is most probably due to a similarity in molecular structure or similarity in the immunologically determinant groups in the molecules of the serum proteins of man, apes and monkeys. The occurrence of cross reactions, however, does not detract from the practical value of the test, as they can be eliminated by suitable methods. An antiserum prepared in an animal whose antigens show cross reactions, for instance antihuman serum from monkeys, does not show cross reaction.

Precipitation may also be caused by certain partial antigens or haptens. An example of this is the capsular antigen of the pneumococcus. An antipneumococcal serum precipitates not only the total pneumococcal substance in solution, but also the pure capsular antigen. Visible flocculation may, however, occur only

when the haptens are of high molecular weight, as in the case of the polysaccharide haptens; with simple haptens, like certain organic acids or monosaccharides, no visible reaction takes place, although the specific hapten-antibody union will have occurred. That such an invisible union may take place is proved by the fact that a precipitating serum treated with a simple hapten loses its capacity to precipitate with the complete antigen subsequently.

Reference has already been made elsewhere about the close similarity between the precipitin and agglutination reactions and how collodion particles covered over with antigen, whereby they come to behave as antigen particles, undergo agglutination like bacteria in the presence of ascending dilutions of the precipitatinig serum. Further, the antigen-antibody complex in a precipitin reaction has been shown to be capable of fixing complement, pointing to the close relationship between all serological reactions.

The precipitin reaction is a very delicate one; a precipitating serum will give visible reaction when mixed with its antigen even when this is diluted several thousand times. This has very important practical significance; it provides a far finer test for the detection of proteins than any chemical test. The precipitin test is employed for various purposes: in medico-legal work to determine the origin of blood stains; in the identification of bacterial types, as in the typing of pneumocci and meningococci; in diagnostic procedures, as in Kahn and Ascoli's tests; in the titration of antiserum and in the detection of fraudulent substitution of food materials.

Agglutination. The phenomenon of agglutination can be demonstrated by mixing together a bacterial suspension and the specific immune serum. First the organisms, if motile, lose their motility and then aggregate into small clusters which soon form large clumps. In the test tube the clumps rapidly settle down to the bottom and the column of fluid, which was turbid at the commencement of the test, is rendered clear. The speed and completeness of reaction are influenced by a variety of conditions which are described below.

While agglutination had been observed by other investigators, the phenomenon of specific bacterial clumping was first recognised by Gruber and Durham in 1896. In the course of their studies on the Pfeiffer phenomenon, they observed that on mixing with

the homologous serum the cholera vibrios in a broth culture clumped together and settled to the bottom, leaving the supernatant fluid clear. It was subsequently found that any foreign cell, such as yeast or erythrocytes, will stimulate the production of agglutinating serum for that cell. Shortly afterwards, Widal and others studied the special application of this phenomenon in the case of enteric fever and established it as a valuable diagnostic aid in this disease. Later on, its application was extended to the diagnosis of many other infections, like cholera, dysentery and undulent fever. Early observers had called attention to the fact that normal sera may agglutinate many bacterial species in low titre, up to 1 : 10. The presence of the so-called normal agglutinins in the blood has been referred to elsewhere. This has to be kept in view when conducting routine diagnostic tests. Haemagglutinins are a type apart and are dealt with separately (p. 274).

The agglutination reaction occurs in virtue of the presence of certain specific substances in the agglutinating serum called *agglutinins*. Their production in the body is stimulated by bacterial or other antigens termed *agglutinogens*. Sera of high agglutinating potency, agglutinating in a dilution of 1 : 20,000 or more, may be prepared by the immunisation of suitable animals. Such agglutinating sera are referred to as *high titre sera*.

This reaction is analogous to the precipitation reaction; it occurs between two interacting substances, antigen and antibody; it does not require the co-operation of the complement, the agglutinating activity not being destroyed by heating the serum at 56° C.; but the presence of electrolytes is essential. The difference between these two reactions is due to the difference in the size of the particles constituting the antigen. It is to be noted that, while in precipitation the antigen is in fine colloidal solution, in agglutination it is composed of relatively large bacterial or other cells, forming a highly complex system often with a number of antigenic components distributed over the cell surface. As in precipitation, agglutination occurs in two distinct stages. In the first stage, there is a rapid interaction between the agglutinin and the bacteria, most likely a process of adsorption; this interaction is specific. It is followed by the second phase in which there is aggregation of the sensitised bacteria through salt action; it is non-specific. The presence of electrolytes in the

medium is, therefore, essential also for the agglutination reaction. In the absence of salt no clumping will take place. This can be demonstrated readily. The interaction is allowed to take place with reagents dialysed free of salt, *i.e.* in a salt-free medium; the mixture is centrifuged and the sediment, composed of the antigen-antibody compound, resuspended in normal saline when agglutination is found to occur without any further addition of the specific serum; the supernatant fluid after centrifugation, on the other hand, is found to be deprived of its agglutinin content. Thus, it is not the agglutinin that actually brings about clumping; its union with bacteria renders them readily flocculable.

In the ordinary pH range bacteria are negatively charged and in an electrical field (cataphoresis) traverse to the anode. As in colloidal suspensions, the like electrical charge of the individual cells is responsible for the maintenance of the stability of a bacterial suspension. Operating against this repelling force is the natural intercellular cohesive or attractive force which appears to be a function of surface tension. But so long as the repelling force is unimpaired, the effect of the attractive force is negligible. In the agglutination reaction this repelling force is overcome and the physical conditions of the bacterial cell so altered as to force out the bacteria from suspension. The factors responsible for this are the specific agglutinin and the electrolyte. There does not seem to have much doubt that the essential part played by the former is to transform the bacteria from a hydrophilic to hydrophobic state. Opinion is divergent whether their sensitisation has also a part in reducing the surface charge. Whatever that be, there is no disagreement on the point that it is the electrolyte that is primarily concerned in overcoming the repellent force. While salt neutralises the electrical charge, it also at the same time decreases the cohesive force. But serum seems to antagonise this action of salt and prevent it from reducing the cohesive force of the bacterial cell. The changes in the physical condition brought about by antibody sensitisation are not alone adequate to cause clumping which, under ordinary conditions of experiment, requires a decrease of the surface charge to a low level of 13–15 mvt. So long as the surface potential is not reduced to this level, the sensitised bacteria remain disperse. The function of the electrolyte is to depress the charge below this critical level when the natural cohesive force

comes into operation, completing the reaction as expressed by visible flocculation.

Several physical factors, such as temperature, pH of the medium and concentration of the interacting substances, influence the rapidity of action in the second phase. The temperature employed in performing the test is 56° C. In most cases normal saline proves quite a satisfactory medium. The bacteria may be living or killed; if alive they are not killed by the reaction. In fact, the agglutinated bacteria may continue to grow although in bizarre forms of long delicate chains—the so-called " thread reaction".

Quite independent of the presence of immune antibodies, acids in certain concentrations have a clumping effect on bacteria. It is probably the concentration of hydrogen ions that is responsible for it. At a sufficiently low pH level, about 3·0–4·0 for most bacteria, which corresponds to the iso-electric point of the bacterial protein, spontaneous agglutination takes place. It is called *acid agglutination*. At one time it was regarded that this pH value was different for different bacteria, having, therefore, a differential value. This view has been proved incorrect by later work.

Spontaneous agglutination is a common feature with certain microbic species, such as haemolytic streptococci, tubercle bacilli diphtheria bacilli and others. It is probably a reflection of greater cohesive power among the cells of these species. A great handicap in practical work, it can, however, be eliminated in many cases by growing the organism at room temperature or by suspending it in low salt concentration or at a buffered reaction of pH 7 to 8. Spontaneous agglutination is also a property of rough variants which agglutinate in salt solution not containing any specific agglutinin (p. 345). The importance of spontaneous agglutination on the practical side is that it may present us with technical difficulties when performing the test.

Cross agglutination is a frequent phenomenon among closely related bacterial species. It is due to a sharing of antigenic components among them. Mention has been made elsewhere that the bacterial cell is not antigenically simple, but composed of a number of serologically different moities (p. 237). Thus, an antigenic component possessed by one species may be common to another allied species. The specific serum produced against

the first will cross-agglutinate the allied one but generally in a low titre. Such common antigens are spoken of as *group antigens* and the agglutinins responsible for cross reaction *group agglutinins* or sometimes *minor agglutinins* in contrast to the strictly *specific major agglutinins* formed against the main antigens. Group agglutination is a conspicuous feature of the *Salmonella* and dysentery groups of organisms. The formation of group agglutinins, however, does not detract from the practical value of the test as a specific one. The minor agglutinins in an agglutinating serum can be put out of action by sufficient dilution.

Prozone Phenomenon. It is a peculiar phenomenon sometimes observed in agglutination and other serological reactions. In this, agglutination takes place in high dilutions of the immune serum, while it fails to cocur in low dilutions, 1 : 10 to 1 : 20, *i.e.* in the first two or three tubes in which the serum is present in the greatest concentration. This zone of low dilution where there is inhibition is known as the *prozone* or *proagglutinoid zone*. The explanation of this is still obscure. The phenomenon is more common with old or heated sera and presumably aging and heating bring about some change in the antibody globulin. This change, at any rate what is brought about by heat, does not seem to do away with the property of specific union; in the prozone the heat-changed agglutinins combine with bacteria, but no clumping follows. Probably, such modified antibody globulins act as protective colloids.

Agglutination Reaction and Bacterial Antigens. The bacterial cell has a complex antigenic structure composed of various components. The importance of this in agglutination reaction has been brought into prominence by recent studies. Whether these various antigenic fractions have any exact distribution on the bacterial body is not clear. This question has some important bearing on the agglutination reaction.

In the case of motile organisms, such as the *Salmonellas*, *Proteus* and others, the flagellar material constitutes a different antigen from that of the somatic or body substance. The former is called the flagellar or H (Hauch = spreading) antigen from the spreading nature of the colonies of these organisms on solid media and the latter the somatic or O (Ohne hauch) antigen as the colonies are not spreading but small and discrete. The H antigen is naturally situated on the surface of the body and

the O antigen deeper to it. Each of these types stimulates its own agglutinin; thus, there is the H agglutinin and the O agglutinin, each interacting only with the corresponding antigen. This difference is of considerable importance in diagnosis and also in prognosis, as the O antibody seems to have some protective value. The H antigen is relatively labile, being destroyed by heat above 70°–75° C. and by the action of absolute alcohol. Among the non-motile organisms, the H antigen is absent as they are aflagellate. In them the superficial antigen is of the somatic or O type. It resists a temperature of 100° C. and the action of absolute alcohol. With H antigen and its antibody agglutination occurs rapidly. The clumps formed are fluffy composed of large loose flakes which consist of bacteria come together and entangled by their flagella. With O antigen the clumps are slow to form; they are small and granular as the bacterial cells are closely packed together. The differences in the physical states of the clumps are purely consequential to the presence or absence of flagella. These structures get entangled themselves, preventing close approach of the bacterial bodies.

The H antigen, like the O antigen, is not always simple in structure. The H antigen of many motile species exhibits what is called *phasic variation.* The flagellar substance of a motile species may consist of one or more antigenic fractions. These components may be highly specific, *i.e.* peculiar to the species or, what is more complicating, some of them may be common to other species. An ordinary culture contains both groups, some bacilli bearing the specific antigens and others the non-specific antigens. Organisms possessing the specific antigens are said to be in the *specific phase* and those that possess the group antigens are said to be in the *group phase.* Strains that display a tendency to such *specific-group phase variation* are called *diphasic strains.* In either phase there may be more than one H antigen. For example, *Bact. paratyphosum B* may remain either in the specific or in the group phase. In the specific phase there is only one antigenic component called b, but this is replaced in the group phase by two different components called 1 and 2 which are also common with the group phase H antigens of *Bact. typhi-murium* (Table XIV). By preparing plate cultures and subculturing individual colonies, pure cultures can be obtained of organisms entirely in the specific phase or in the group phase.

The former is very unstable and may soon give rise to progeny, some exhibiting specific and some group characters. In the specific phase the antigen is highly specific and a culture containing the specific phase type agglutinates only with the serum prepared against the specific phase culture, whereas in the group phase, as the antigenic components are shared by a large number of allied species, cross agglutination occurs. It must, however, be noted that this separation into specific and group phases is not sharp (p. 348).

The O antigen does not show any tendency to phasic variation like the H factor but remains fairly constant within the species. In the same species, however, there may be more than one O antigen. For example, *Bact. paratyphosum B* contains four somatic antigenic components, I, IV, V and XII. There may also be common O components among closely allied species (Table XIV). This is a striking feature of the *Salmonellas*.

The smooth somatic antigen does not remain constant under all conditions. The S-R variation occurring in bacteria is also associated with changes in the antigenic structure and loss of specificity. The rough variant does not contain the specific O antigen. The non-specific rough antigen now exposed is called the R antigen and is distinct from the O antigen. With this transformation, the H antigen is not necessarily lost and the rough variant may remain motile, *i.e.* there can be a rough motile strain.

Labile Antigens. Attention has been recently drawn to the presence of certain antigens, apparently on the surface, in some bacterial species, which are readily destroyed by heat and certain chemicals and for this reason called *labile antigens*. Because of their instability, earlier workers missed their existence. The Vi antigen of the typhoid bacillus is an example. This antigen is associated only with virulent strains and it appears to be of importance in active immunisation (Chapter XX).

Agglutinins. The agglutinating antibody is called *agglutinin*. The different types of antigen mentioned above give rise to the formation of corresponding agglutinins in the body. Thus, there is the H agglutinin, O agglutinin and Vi agglutinin. They interact only with the homologous antigens. While the H antigen is relatively labile, the H agglutinin is relatively stable; the O antigen is stable, whereas the O agglutinin is relatively labile. It has been mentioned on a previous occasion that the H agglu-

tinins have little protective value, while the presence of O and Vi agglutinins in the serum has been associated with increased resistance. But the occurrence of in vivo agglutination has not been definitely proved. Admitting that it happens, we may surmise from the effect of in vitro reaction that agglutination may not directly cause the destruction of the agglutinated bacteria; it may render them more readily vulnerable to other defence forces.

Application of the Agglutination Test. The agglutination test is of considerable value in the diagnosis of infectious diseases. It is made use of in two ways: in one, the presence of a specific agglutinin in the patient's serum is determined by the employment of known bacterial antigens; in the other the reaction enables to establish the identity of an organism which is isolated from a patient with the help of known sera. The agglutination reaction is also employed in the typing of bacteria, for example the typing of streptococci or pneumococci.

The test is conducted in two ways: the microscopic and the macroscopic. In the former a bacterial suspension is mixed with the serum dilutions on the slide and examined after half to one hour under the one-sixth inch lens of a microscope. This is a much less satisfactory method than the macroscopic method. In the latter the test is done in small tubes arranged in suitable racks. Serial dilutions of the serum—1 : 10 upwards to any desired extent—are made with physiological salt solution, maintaining a constant volume (0·5 or 1·0 ml.) in each tube. An equal volume of the bacterial suspension, standardised to contain a certain number of organisms, is added to each tube. This doubles the serum dilutions. The rack is then incubated in a water bath at 56° C. for two hours or at 37° C. overnight and the readings are taken two hours after removal from the water bath or the following morning. The highest dilution of the serum at which there is visible clumping is reported; it is taken as the end titre of the serum.

In the conduct and interpretation of the test certain difficulties have to be overcome and fallacies avoided. Particular attention should be paid to the condition of the culture used—somatic antigen, flagellar antigen or labile antigen. The possible occurrence of spontaneous agglutination, particularly evinced by rough variants, should be remembered. Some organisms, for instance, Sonne's dysentery bacillus, undergo rapid dissociation.

In their case only freshly isolated strains should be employed in the test. It may be reminded that different species and even different strains may exhibit differences in agglutinability; this has to be met by suitably modifying the technique. In the identification of organisms by agglutination, specially prepared high titre sera should be employed in order to eliminate errors from group agglutinins. The presence of labile antigen in the culture used may prevent agglutination by O antibodies. Complication may likewise arise from the fact that a patient's serum may contain group agglutinins, normal agglutinins or residual agglutinins after prophylactic immunisation. This and cognate problems are discussed under the diagnosis of enteric infections.

Agglutinin Absorption. Many bacterial species exhibit a heterogeneous antigenic structure and consequently stimulate the production of multiple antibodies which are not all specific. It follows that the antiserum produced against any such species will contain a number of agglutinins. The method of agglutinin absorption provides a means of demonstrating these facts and establishing the antigenic relationship of bacteria; it further helps in the disentanglement of the antigenic mosaic of a bacterial cell through a process of selective absorption of agglutinins. The union between the agglutinin and the agglutinogen is a fairly stable one. By treating an agglutinating serum, usually diluted (1: 50), with a dense suspension of the homologous organism, incubating for two or three hours and removing the agglutinated mass by centrifugation, it is found that the serum has been deprived of the corresponding agglutinins; the organism has absorbed the agglutinins and removed them from the system. Of course, it is not so simple a process and in practice it is necessary to absorb repeatedly two or three times and even then complete absorption may not occur. Allusion has been made elsewhere that some of the antigenic components, and consequently antibodies, are common to biologically related species. Those bacteria that share antigens also develop group agglutinins (minor agglutinins) for related species besides their own major agglutinins (p. 268). From such an antiserum the homologous bacterium will remove both the specific, or major, as well as the group agglutinins, whereas the related species will remove only the group agglutinins, leaving the major agglutinins intact. Again, the group agglutinins and the specific agglutinins formed by

a bacterium are sometimes found to be almost of the same titre. In these circumstances, the members of an allied group cannot be differentiated by simple agglutination tests which will give only equivocal results; the agglutinin absorption test will have to be done in such cases for final differentiation.

Haemagglutinins

Agglutinins that cause clumping of red blood corpuscles are called *haemagglutinins.* They occur naturally or can be artificially induced by the injection of red cells. But the term haemagglutinins is usually employed to denote the former. Natural haemagglutinins occur in human sera; similarly, agglutinable substances, *haemagglutinogens*, are found in the erythrocytes. As the former act on the cells of the same species, they are called *isohaemagglutinins* or simply *isoagglutinins.* Associated with these agglutinins may also be found haemolysins; lysis takes place in vivo but only rarely in vitro. These agglutinating and agglutinable factors form part of the physiological make-up of man, but in the normal individual no agglutination takes place due to differences in their distribution.

For much of our knowledge about the subject of blood grouping we owe to Landsteiner. He classified (1900) human blood into three groups; this was soon followed by the addition of a fourth group by his associates. But it should be mentioned that Jansky (1907) and Moss (1910) independently described the same four groups but gave them different notations. It is Landsteiner's notation, adopted as the international nomenclature, that is now employed everywhere.

Landsteiner demonstrated the existence of two types of agglutinins which he called *a* and *b* and two types of corpuscular agglutinogens A and B and further showed that one type of agglutinin is never found in association with the corresponding type of agglutinogen. Further, both agglutinins may be present in the same individual in which case no agglutinogen is found at all in his cells; or both cell substances may occur together when no serum factors are present. This peculiarity in the distribution of the interacting substances explains why no agglutination occurs in the normal state.

According to the distribution of these factors in the blood, humanity can be classified into four large groups, designated

O, A, B and AB, indicative of the contained cell factor. The following table shows the interrelationship between the cells and sera of different groups:

TABLE III
Human Blood Groups

Cells of group	Serum of group			
	O *a* and *b*	A *b*	B *a*	AB *o*
O	−	−	−	−
A	+	−	+	−
B	+	+	−	−
AB	+	+	+	−

+ = agglutination; − = no agglutination.

Later investigations showed that the A agglutinogen is separable into two subgroups, A_1 and A_2, giving rise to a total of six groups, O, A_1, A_2, A_1B, A_2B and B. A_1 and A_2 differ widely in their sensitivity, A_1 being more sensitive than A_2. When associated with the B substance, the reactivity of A is weaker than when present alone. The A in A_2B is the feeblest so that an A_2B blood may be missed unless a potent anti-A serum is employed. In adults the B factor evinces little or no variation in reactivity.

Group sera for typing should be obtained from young healthy adults who show a high titre of agglutinin content in their serum. The agglutinin titre of very young children or old people is usually lower than that of individuals of other age groups. Sera that give reactions visible to the naked eye in a minimum dilution of 1 : 40 are satisfactory. They are heated at 56° C. for 20–30 minutes, put in vials of 1 c.c. capacity, labelled and stored in the refrigerator. Thus preserved, they retain their potency over long periods. Addition of preservatives is not necessary; if desired, 0·5 per cent. phenol may be employed. Strict aseptic precautions should be taken during preparation and subsequent manipulations. It is advantageous to dilute the group sera with an equal volume of isotonic saline solution as it avoids rouleaux formation. Grouping can be done with A and

B sera alone, but it is preferable to keep also a stock of O serum. Group sera are also available in certain commercial firms.

The grouping of blood is a relatively simple matter. It may be done in short narrow test tubes or by the simple slide method which is in more common use. As it prevents evaporation, the hanging-drop method may have advantage over the ordinary slide method. As far as possible, fresh cell suspension should be used, since preserved cells cause anomalous reactions. A 1–2 per cent. suspension is the proper concentration. A drop of blood from a finger puncture is transferred to 2·5 c.c. of a 1·5 per cent. sodium citrate solution in physiological salt solution. It gives approximately a 2 per cent. suspension. As the presence of citrate does not interfere with the reaction, blood may be taken in citrate saline and directly used for grouping. If desired, the cell suspension may be centrifuged and the deposit resuspended in normal saline in the required concentration. A drop of the unknown cell suspension is deposited on a clean labelled slide side by side with a drop of the standard serum. The two are mixed and the slide rocked back and forth. The use of a cover-slip is not necessary, unless examination by the microscope is contemplated. Definite agglutination occurs at the laboratory temperature but is hastened at 37° C. Macroscopic clumping will be evident in a few minutes (5–10 minutes) in positive cases; a hand lens may help in its detection. A positive reaction may at times be slow to develop and final reading should not be taken before at least fifteen minutes. Thus, the unknown cell can be quickly tested with the three group sera and its group can be readily determined from the following scheme:

TABLE IV

Group A Serum	Group B Serum	Group O Serum	Group of the unknown cells
+	+	+	AB
−	+	−	A
+	−	+	B
−	−	−	O

+ = agglutination; − = absence of agglutination.

False reactions should be carefully guarded against. Deterioration of the standard serum may be a source of false negative reactions. It is to be noted that the reaction may be considerably delayed if a concentrated cell suspension is used; hence, results should not be read hastily. Weak reactivity of cells may be another source of false negatives. The sensitivity of the agglutinogens of the new born infant is low, but no reliance is placed on group determined at this age. Preserved blood sustains a progressive diminution of sensitivity. As mentioned above, A_2B is a weak reactor and considerable experience and care are necessary to diagnose it. With fresh and highly potent sera haemolysis may occur rarely, masking agglutination; but this can be easily made out. False positives may be due to pseudo-agglutination or to bacteriogenic agglutination (see below); they may also be caused by the presence of autoagglutinins or atypical isoagglutinins in the test sera (p. 280).

Pseudoagglutination is due to rouleaux formation, the cells arranging themselves like piles of coin, and, when it is marked, gives the appearance of true clumping. The phenomenon is common with the blood of patients suffering from acute infectious diseases and also of pregnant women; their sedimentation rate is also rapid. The rouleaux formation is due to changes in the viscosity of serum and is correlated with an increase in the protein content of blood, especially fibrinogen. But the exact nature of the substances responsible for the reaction is not clear; the active agent cannot be absorbed out as in the case of isoagglutinins. Also, the reaction is not specific and the particular serum will cause rouleaux formation with any red cell suspension. Under the microscope rouleaux can be easily made out from true clumping. The property disappears on slight dilution of the serum; even from the already formed clumps the dispersal of cells may often be effected by adding a drop of normal saline.

Bacteriogenic agglutination is due to changes in the sera or cells resulting from bacterial contamination. It can be avoided by using only uncontaminated sera and fresh cell suspension.

Apart from the red cells, the group antigens are also present in the cells of most of the organs and in the spermatozoa (pp. 236, 252). The cells of tumours, both benign and malignant, exhibit the property of absorbing isoagglutinins. The body fluids and secretions likewise contain the group-specific substances; thus, they

are present in the serum, saliva, semen, sweat, tears, urine, milk and digestive juices but not in the cerebrospinal fluid. Isoagglutinins have been demonstrated in the amniotic fluid, but their presence in the placenta is doubtful. The presence of extracellular group substances in the body has been suggested to be a protective mechanism against damage in mother and foetus in heterospecific pregnancies, *i.e.* pregnancies with blood group incompatibility between mother and foetus.

The group characters are inherited according to the Mendelian laws of genetics. The existence of three pairs of allelic genes, A, B and O with A and B as dominant to O, has been postulated. Their transmission is not influenced by sex. On the hereditary nature of blood groups is based the application of blood grouping in forensic medicine and anthropology. Though hereditary, the group characters are only imperfectly developed at birth. Isoagglutinogens are present in the erythrocytes of the newborn, but their reactivity is considerably weaker than in adult life. Full development of reactivity takes place during the process of normal growth and groups are established within the first two years of life. Whatever agglutinins are present at birth are derived from the mother's blood. They disappear in the course of a few weeks and three to six months after birth the infant begins to develop its own natural agglutinins. Once established, the group remains constant throughout life. There is no proof that diseases exercise any influence on blood groups. Apart from variations in the isoagglutinin content among individuals, the titre of these natural antibodies may vary with the age of the individual, in certain diseases and even in the absence of any traceable cause. The sexes do not display any appreciable difference in their isoagglutinin titres.

Three more isoagglutinogens have been discovered and named M, N and P. No corresponding antibodies against M and N are naturally found. The presence of these antigens, therefore, is not of any consequence in blood transfusion. They are not related to the A and B cell factors and their distribution is independent of the major groups or sex. According to the presence of M, N or both M and N, three distinct blood groups, M, N and MN, are defined. Absence of these factors have never been encountered in any blood. M and N factors are also inherited strictly according to Mendelian laws. Hence,

they are also of value in forensic medicine in the exclusion of parentage in disputed cases. The P factor also follows the same genetic laws as the M and N factors and are likewise distributed in both sexes and with no reference to other groups. Antibodies against P are only very rarely encountered in normal sera or after repeated transfusion.

Importance of Blood Grouping. The discovery of blood groups, apart from its academic interest, has placed in our hands knowledge of manifold interests. It finds practical application in many fields of work. It has proved its unquestionable importance in transfusion therapy. In forensic medicine its value in the identification of blood stains and for the determination of non-paternity has been well attested. Its contribution to the study of anthropology has been immense. The blood groups provide an excellent means of studying heredity in man, regarding which our present knowledge is poor compared to that about plants and animals. The study of haemagglutinins has considerably expanded our knowledge of the nature of cellular antigens. The subject, therefore, is of much wider interest than to the clinician and to the immunologist.

As noted above, blood grouping is of supreme importance in blood transfusion, as it helps to prevent serious and fatal reactions following transfusion. In causing reaction it is the cells of the donor that matter; the relatively small quantity of the donor's serum will be quickly and sufficiently diluted by the recipient's blood and thus prevented from exerting any appreciable effect on the recipient's cells. The O group contains no antigen in their cells, thogh it has both *a* and *b* agglutinins in the serum; consequently, their cells will not be agglutinated by the serum of any recipient. Hence, this group is called the universal donor. Group AB is the opposite of it and is called the universal recipient. It is relatively safe to transfuse from a donor belonging to the same group as the recipient or from a universal donor. What is essential in transfusion is that the recipient's serum should not clump the donor's cells, and to minimise the risk still further a direct matching should always be done prior to transfusion. It can be done in a very short time (see above). Intragroup incompatibility, rarely encountered, may thus be guarded against.

In recent years, grouping has also acquired increasing importance in medico-legal work. It helps to exclude the paternal

parentage in disputed cases and also in the identification of blood stains. As the transmission of these natural antigens and antibodies from parents to progeny follows strictly certain genetic laws, parents can transmit to their offspring only those characters which they themselves possess. The presence in the child of a group substance, which is not found in either the mother or the alleged father, exonerates the latter from that child's paternity. For example, if both parents belong to group A, no children of group B or AB can be born of them. A+A will produce only A but not B or AB. Obviously, in no case can it be established that a certain individual is the parent of a particular child. Thus, it is a negative evidence; nevertheless, it is of great value not only in controversies involving questions of paternity, but also in those centering round the identity of a child when doubt arises as the result of interchange in the infants' wards accidentally or intentionally.

Blood grouping has proved to be a valuable aid to the study of anthropology. Because of the relative simplicity of technique, not inconsistent with reliability, blood tests have been extensively used in recent years in this field of work. They have provided very reliable data regarding the geographical distribution of races, the racial origin of various groups of population and other kindred problems.

Autoagglutinins. When the serum of an individual agglutinates his own cells, the phenomenon is called *autoagglutination*. It was considered to be a purely pathological phenomenon, but this view had to be revised. Normal sera often show the presence of autoagglutinins. Generally, autoagglutination occurs only at 0°–5° C. and, as the temperature is raised, reversal of clumping follows. Autoagglutinins are non-specific and the serum of any individual containing these antibodies will clump the cells of any other person irrespective of group, leading to the conclusion that the specific agglutinogen is present in all human bloods. Unlike pseudoagglutinins, they can be removed by absorption, resembling in this respect isoagglutinins. In certain pathological conditions, like paroxysmal haemoglubinuria, syphilitic and hypertrophic cirrhosis of the liver, haemolytic jaundice, Raynaud's disease, severe anaemias and trypanosomiasis, the autoagglutinin content of serum increases considerably and agglutination may occur even at room temperature. But it is very rare.

The presence of autoagglutinins in sera, which react at room temperature, is another source of fallacy both in grouping and cross matching. Unwashed cells of the patient may be clumped by any test serum and he may be grouped as AB. In doubtful cases, therefore, cells washed with warm salt solution are to be used for the test which should be conducted at 37° C., at which the autoagglutinins are not active. Difficulty in cross matching can be got over by removing autoagglutinins from the patient's serum through the agency of his own cells by keeping the blood at 0°–5° C. and by using only washed cells of the patient.

Autoagglutinins should not be confused with atypical or irregular isoagglutinins that may very rarely occur in normal human sera. Mostly, these irregular agglutinins are active only in the cold when they are referred to as *cold agglutinins*. Their titre is too low to interfere with the grouping of blood.

Rh factor. The Rh factor is a minor isoagglutinogen discovered in 1940 by Landsteiner and Wiener. Its presence was first demonstrated by them in the rhesus monkey, in which it was found to be a constant factor. Hence the name *Rh factor* was assigned to it. Shortly after, it was shown to be also a constituent of human erythrocytes. In contrast to the major group substances, no natural agglutinin against the Rh factor exists, but it is induced by active immunisation. The distribution of the Rh factor among the several races has not been extensively worked out. About 85 per cent. of the Anglo-Saxons have it; the rest of them are Rh-negative. Limited search indicates that the frequency is very much higher among the American Indians (92 per cent.) and the Chinese, among whom there are practically no Rh-negatives.

The serum of the mother of an infant with erythroblastosis foetalis is the most dependable source of anti-Rh agglutinins. Patients (Rh-negative) who have had haemolytic reactions after repeated transfusions from donors (Rh-positive) of the same group also yield potent anti-Rh sera. Such sera can be prepared in rabbits, or more easily in guinea-pigs, by immunising them with rhesus erythrocytes. It would appear that anti-Rh lysins are also formed, but for some unexplained reasons these act only in vivo and not in vitro. Serum from any source may be used for grouping, but the major agglutinins, if present, should be removed by the agency of A and B substances. Human sera

should be inactivated at 56° C. for 10–20 minutes; otherwise, reactions may be suppressed. Compared to the reactions which determine the major blood groups, the Rh agglutination reactions are feebler. The test is best carried out in short narrow tubes; as it gives far more accurate results than the slide method. It is usually done at 37° C. and the results are read after about an hour both macroscopically and microscopically. Rh agglutinins deteriorate rapidly; they also decline soon in the body. Reports about their occurrence in the general body fluids and tissue cells are conflicting.

The practical importance of the Rh factor, apart from its academic interest, was demonstrated almost immediately after its discovery. Its role in the causation of erythroblastosis foetalis has been generally accepted now. Though its antigenic potency is of a lower order than that of the major group substances, nevertheless, it stimulates the production of specific agglutinins in the Rh-negative individual. The Rh quality is inherited according to strict genetic laws. An Rh-negative woman mated with an Rh-positive husband may have an Rh-positive foetus (heterospecific pregnancy). The Rh property develops early in life. If through any flaws in the placenta the foetal erythrocytes gain entrance into the maternal blood stream, anti-Rh agglutinins are incited in the maternal tissues and discharged into the circulation. They are transmitted across the placenta to the foetal circulation in large concentration, probably a few days prior to parturition, or, after birth they are absorbed from the mother's milk. In the circulation of the newborn specific antigen-antibody union ensues, leading to haemolysis of varying degree, consequent anaemia and jaundice. This condition constitutes erythroblastosis foetalis or the haemolytic disease of the new born.

The discovery of the Rh factor is also of considerable practical significance in transfusion. Severe reactions that occur occasionally after transfusions of homologous blood are some of them due to the introduction of Rh cells into the Rh-negative recipients. Such reactions are more common after repeated transfusions or after a first transfusion after labour or still birth.

The Rh factor begins to appear early in foetal life and is fully developed at birth, after which it remains constant. It has no relation to the A, B, M and N factors but occurs independently of them. The Rh property is not sex-linked but is distributed alike in

both sexes. According to Landsteiner and Wiener, it is inherited as a Mendelian dominant through a pair of allelic genes, *Rh* and *rh*.

Subsequent work shows that the Rh factor is not a single simple antigen; it represents a group. With the aid of sera containing anti-Rh agglutinins, three well defined types of agglutinable factors, Rh_1, Rh_2, Rh_{12} (Rh_1, Rh_2), have been recognised. These factors occur in the blood cells either singly or in combination, making up at least five different antigenic complexes. Possibly there are others also. By means of the three types of anti-Rh sera, human beings can be divided into eight Rh groups including the Rh-negative type. Analogous to the Rh factors but qualitatively different from them is yet another antigen called the Hr factor. The corresponding agglutinin is in its activity the reciprocal of the standard anti-Rh agglutinin.

Bacteriolysins and Bacteriocidins. These are antibacterial antibodies developed in the course of both infection and immunisation. Acting on certain bacteria they cause lysis or dissolution when they are referred to as *bacteriolysins*. On others their effect is simple killing, unassociated with gross degenerative changes; in such cases they are referred to as *bacteriocidins*. As lysis obviously implies death, we are dealing with destructive effect in either case and in practice no differentiation is necessary between the two.

Early investigations revealed that fresh defibrinated blood of animals possessed lytic properties. Subsequently it was shown that fresh serum retained this property, signifying that the lytic action was not due to any cellular activity. Sera obtained from different animal species were found to differ widely in their lytic action towards the same species of bacteria; also, the blood or serum derived from the same animal source was not found equally lytic to different species of bacteria. The discovery was then made that the lytic effect was destroyed by heat at 56° C. for thirty minutes, suggesting that it was dependent on some labile factor naturally present in the serum. This labile factor was called *alexin* by Buchner and later complement by *Ehrlich*. Later it was shown that not only bacteria but also erythrocytes were susceptible to the lytic action of normal serum. Lysis in all such cases was found to occur only in low titre.

Antibacterial substances are provoked by bacteria in a specific manner and are present in the serum. Such sera are called

immune sera. They exhibit a high degree of lytic and killing properties. As mentioned above, visible lysis is not an invariable result of the lethal action of an immune serum. It also depends on the nature of the bacterial cell. The majority of bacteria are not dissolved, but it is not due to any failure of combination with antibody and complement. The Gram-negative bacteria appear to be more susceptible to the lytic changes than the Gram-positive ones. The lytic action of bacteria is best demonstrated by the in vivo method of Pfeiffer. He was the first to observe this phenomenon with the immune serum; hence it is called *Pfeiffer's phenomenon.*

Pfeiffer's Phenomenon. A guinea-pig is immunised against *V. cholerae.* Then it is injected intraperitoneally with a suspension of the same organism. At varying intervals after it, small quantities of the exudate from the peritoneal cavity are aspirated and examined by the hanging-drop method. The vibrios first become immobile, then show swelling, granulation and complete disintegration, leaving no trace of the bacterial cell. Similar changes are not seen in the organisms inoculated into a normal animal or seen only in a low degree. But they can be obtained by injecting a normal animal with a mixture of the vibrio suspension and immune serum, even when the latter has been heated at 70° C. for one hour. What is destroyed by heating (complement) is made good in the guinea-pig's peritoneum.

The lytic phenomena also occur in vitro. The serum of a guinea-pig immunised against *V. cholerae* is powerfully lytic to these organisms. The lytic activity is destroyed on heating at 56° C. for thirty minutes. This process is called *inactivation* of serum. The addition of fresh non-immune guinea-pig's serum restores the lytic property of the heated serum, *i.e.* reactivates it. Fresh normal guinea-pig's serum alone is not lytic or only feebly so. Thus, heated immune serum and fresh non-immune serum are both necessary for interaction. This may be illustrated by the following scheme:

TABLE V

V. cholerae + fresh immune serum	lysis
V. cholerae + heated immune serum	no lysis
V. cholerae + fresh guinea-pig's serum	no lysis
V. cholerae + heated immune serum + fresh guinea-pig's serum	lysis

This clearly shows that the fresh immune serum contains two factors both of which are essential for producing the lethal effect. One is a relatively heat stable factor and is called *immune body* or *lysin*. The other is the heat labile alexin or complement. First the immnue body combines with the antigen; and secondly the antigen-antibody complex so formed unites with the complement, leading to a completion of the reaction. The first part of the reaction is highly specific. If, in the first or last experiment of the protocol, *V. cholerae* is substituted by *Bact. typhosum* or any other soluble bacterium, no lysis will occur. On the other hand, the action of complement is non-specific.

Haemolysis. Haemolysis means a laking of the red blood corpuscles or the liberation of the contained haemoglobin into solution; the stroma does not dissolve. It occurs under the influence of a number of agents, such as immune haemolysin, natural haemolysin and bacterial haemolysin, but we are concerned here with the immune haemolysin, also called the *haemolytic amboceptor.*

Bordet's investigations with erythrocytes showed that the lysis of bacteria by an immune serum was not an isolated phenomenon but only a special case of a general biological law. Various types of cells, like spermatozoa and cells from different organs, were subsequently found to be capable of stimulating the production of immune bodies, *cytolysins*, and reacting with them in an identical manner as bacteria and erythrocytes (p. 229). But, of all such types of cells, the erythrocytes have been the most studied group, as haemolysis, unlike the lysis of bacteria which is more difficult to observe, affords a simple macroscopic evidence of the interaction. And studies on the mechanism of haemolysis contributed largely to our knowledge of the mechanism of cytolysis in general. Also, in another way was the discovery of the phenomenon of haemolysis of very great importance; it was soon exploited for its application as an indicator of other antigen-antibody reactions in which no visible changes occurred.

General Properties of Immune Body or Lysin. These are the same as those of antibodies in general. The chemical nature of immune bodies is not definitely known. They are moderately heat stable, withstanding a temperature of 60°–70° C. for thirty minutes. Phenol and glycerol do not cause their destruction

and are commonly used as preservatives. Acids and alkalies, especially the former, readily destroy them. Lysins withstand the action of contaminating bacteria for long periods. Being true immune bodies, they display a high degree of specificity. Their activity is also dependent on complement. For fuller information refer to the section on antibodies.

The lytic action involves the interaction of three components: the cellular antigen, immune body and complement. The lytic antibody contained in the immune serum specifically sensitises the cellular antigen when the resulting complex absorbs or fixes the complement, the second part of the reaction being non-specific in that the complement may be fixed by any sensitised antigen. The specific antigen-antibody union may take place even at 0° C. but more rapidly at 37° C. To a certain extent this union is reversible. The union of complement, on the other hand, does not occur at 0° C. but does so readily at 37° C.; unlike the specific antigen-antibody union, it is firm. No visible changes are produced indicative of the antigen-antibody union, but absorption of complement by the sensitised cells results in their lysis which is observable. The order of union between the interacting reagents is obviously important; only after the combination of immune body with the corresponding antigen does fixation of complement occur. Further, complement cannot

TABLE VI

1.	Red cell suspension + immune serum	no action
2.	Red cell suspension + complement	no action
3.	Red cell suspension + immune serum + complement	haemolysis
4.	Deposit from 1 + complement	haemolysis
5.	Supernatant from 1 + cells + complement	no action
6.	Deposit from 2 + immune serum	no action
7.	Supernatant from 2 + cells + immune serum	haemolysis

combine with the antigen or the antibody independently of the other, but union between the last two will occur irrespective of the presence or absence of complement. This can also be demonstrated in the following way: mix all the three components in the proper proportion at 0° C. At this temperature, the specific combination of antigen and antibody does occur but not the complement absorption. Centrifuge the mixture, resuspend the cellular sediment in physiological salt solution and add fresh

complement when haemolysis occurs at 37° C. The supernatant fluid will show the complement.

Mechanism of Lysis. Ehrlich postulated that the mechanism of union is a linkage through chemical groups held by the immune body. Complement, according to him, is not directly attached to the cell but only indirectly through the intervention of the immune body which holds, as it were, the antigen on the one hand and complement on the other. In order to convey the idea that this type of antibody possessed two combining bonds, he coined the term amboceptor (twin-receptor) (p. 246). Opposing this view, Bordet enunciated that the whole mechanism of reaction is of the nature of a specific adsorption in which the cell antigen is primarily sensitised by the immune body in a specific manner and is rendered susceptible to the lytic action of the complement (p. 250). He called the lytic body sensitiser. Available evidence supports Bordet's theory. An interesting observation in this connection is that colloidal silicic acid may act as sensitising agent in place of haemolysin.

The Neisser-Wechsberg Phenomenon (Complement Deviation). These workers observed that when immune serum, antigen and complement were mixed, interaction occurred only when the amount of immune serum bore a certain definite proportion to the complement. In those tubes containing larger amounts of immune serum beyond optimal proportion, lysis did not occur. This inhibition of lysis was explained by postulating that in the presence of an excess of amboceptor the complement behaved unusually by combining directly with the free or excess amboceptor in preference to the sensitised cells. The complement was thus deviated from the antigen-antibody complex. But the real explanation is obscure. It may be similar to what is observed in precipitation and agglutination reactions, where there is a failure of antigen-antibody union in the presence of an excess of antigen or antibody.

Natural Lysins. Several types of natural lysins may occur in the blood of man. Their genesis and purpose are both obscure. They may be lytic to the red cells of individuals of other species (heterophile haemolysins or heterolysins) or to various bacteria. Human serum shows lytic activity to the erythrocytes of sheep, rabbits, guinea-pigs and certain other animals. The anti-sheep lysin, and agglutinin which is co-existent with it, may increase

in serum sickness and acute infectious mononucleosis. Isohaemolysins are natural lysins found in association with isoagglutinins, which are lytic to the cells of the same species (p. 274). Autolysins are haemolysins developed in an individual against his own erythrocytes, for example in paroxysmal haemoglobinuria, syphilitic hepatic cirrhosis, trypanosomiasis and others. All the above mentioned lysins dissolve the erythrocytes in conjunction with complement and they are, obviously, special types of cytolysins. Then, there are the bacterial haemolysins whose action is independent of complement. How snake venom disintegrates the red cells is not definitely known. The action of water and other chemicals is purely physical.

Complement. Complement is a thermolabile non-specific substance normally present in the blood of all animals including man. It is not increased by immunisation. It has the property of combining with any sensitised antigen and is most probably responsible for the end result of lysis. The proof of the combination is provided by the ensuing lysis. Per contra, the presence or absence of complement can be demonstrated by the agency of any antigen-antibody complex that can fix complement; most easily it is done with sensitised cells. The union of complement with the sensitised antigen is firm and little dissociation occurs.

Although complement is present in all animals and man, quantitative and qualitative variations are both encountered in different species. Even among animals of the same species fluctuation in the complement titre is common and, indeed, in the serum of the same individual variations occur at different times of the day. But the guinea-pig's blood contains it not only in a high concentration but with great constancy as well. Hence, it is the usual source of complement. Normal sera of guinea-pigs may, however, exhibit qualitative variations. It is because of this that pooled sera are employed in the complement-fixation test. Complement does not exhibit any species specificity and lysis is brought about equally well by complement from different animal species.

The origin or source of complement is obscure. Disintegrating leucocytes have been suggested as its source. There is overwhelming evidence to show that it is a definite chemical entity and not merely a quality of the normal serum. Its true nature is likewise unknown. Complement is intimately associated with

the serum proteins. It is destroyed by trypsin and it has antigenic property. These have led to the belief that complement itself might be protein in nature. It must be mentioned here that the association of complement with globulins and albumins has been recently questioned (*vide infra*). More mysterious is its intimate mechanism of action. It is not analogous to ferment action as a fixed quantity of complement can act only once; its action also depends greatly on its concentration as well as its absolute amount. Evidence of its identity with opsonin is conflicting and these two actions do not always run parallel (*vide infra*).

In the earlier sections, we have seen various immunological reactions in which complement is involved. This naturally raises the question whether there are many kinds of complement or only one. There is no evidence to support the former suggestion; the complement concerned in haemolysis appears to be the same as that concerned in bacterial lysis.

The instability of complement against heat has been referred to above; violent shaking also inactivates it. But in both cases complete destruction does not happen and on standing some activity is regained. Compelment deteriorates rapidly at ordinary temperature (in two or three hours) but resists cold fairly long; hence it can be preserved in the frozen state for several days. It is irretrievably destroyed by acids and alkalies, more easily by the former. Complement activity is suppressed in hypertonic salt solution, but it is restored on reducing the dilution to normality. A method of preserving complement is to raise the salt concentration to 5–10 per cent. and to keep it in the ice chest. Even after several weeks, the potency is restored unimpaired on dilution with distilled water.

The fixability of complement is the highest in the freshly separated serum. After standing for sometime, it becomes somewhat diminished but stable in its titre. But owing to the rapid deterioration of complement at room temperature, more rapid in the hot countries, it should be used not long after the withdrawal of blood, unless it has been kept in the cold storage. The titration of complement is mentioned along with that of haemolysin.

Complement is not a simple, single substance. Guinea-pig's complement has been split up into a number of fractions: the mid-piece, end-piece, third and fourth components. The former

two are dissociated when globulin is precipitated from solution. Neither the supernatant fluid containing the albumin nor the globulin, resuspended in isotonic salt solution, exhibits any complement action. But when they are recombined, the activity is restored. The fraction attached to the globulin is called the mid-piece and what remains in solution, probably attached to the albumin, is called the end-piece. Each acts only in the presence of the other. Mid-piece directly combines with the sensitised cells, although no lysis follows. The end-piece cannot combine directly with the antigen and antibody; only through the mid-piece can it unite with the sensitised antigen and when so bound it produces lysis. Thus, it is the mid-piece that is mainly concerned in the actual complement fixation. Both pieces are thermolabile, being destroyed at 56° C. The third component is inactivated by yeast and also by cobra venom. It is attached both to the mid- and end-pieces, but most to the former. It is heat resistant. The absence of complement activity of some guinea-pig sera is due to the absence of the third component and such sera can be rendered active by the addition of fresh serum from certain animals. This indicates a qualitative difference in the complement action of different species. The fourth component is inactivated by dilute ammonia or viper venom; shaking with chloroform or ether has the same effect. It is also heat stable. Yeast does not absorb it. Though essential for lysis, its absence from fresh serum does not interfere with its opsonic activity, suggesting that complement and opsonin differ at least in some points.

The complement content of blood is the lowest shortly after birth and increases markedly during the first three months of life. Human complement has likewise four components in its constitution. The cerebrospinal fluid contains only the mid-piece. Wide fluctuations have been reported in the complement titre of blood in disease. In every case of serum reaction in which complement is involved, the serum under test should be inactivated so that the complement contained in it may not interfere with the test.

Complement-Fixation Test or Bordet-Gengou Reaction. We have seen that neither antigen nor antibody possesses the property of independent absorption of complement except in a slight degree. But when they unite, the resulting complex acquires this property.

In any immunity reaction involving complement, the specific combination of antigen and antibody should precede the fixation of complement. This sequence is essential, no fixation of complement taking place without this prior union of antigen and antibody. Though the first part of the reaction is specific, the subsequent absorption of complement is non-specific.

The fixation of complement is a general property of the sensitised antigen. Not only cells but also non-cellular antigens, like egg albumin and horse serum, may absorb complement in the presence of the homologous sera. In the case of cellular antigens the interaction may or may not lead to lysis. Where lysis does not occur and where the antigen is non-cellular, there are no observable changes indicative of the antigen-antibody union and subsequent fixation of complement. This difficulty was solved by Bordet and Gengou by employing sensitised red cells as an indicator of the absorption of complement by the antigen-antibody complex. In other words, they employed the sensitised cell for the detection of free complement in a reacting system. Its advantage, that it gives a naked eye proof of lysis, is obvious. Thus was evolved the complement-fixation test. The antibody, when functioning in this way, is sometimes referred to as the *complement-fixing antibody*. It is erroneous to apply the term complement-deviation test to this reaction (p. 287). The name *haemolytic system* was given to the specifically sensitised erythrocytes (*i.e.* the indicator system) and it may be noted that this system is but another complement-fixing system. The haemolytic system usually employed consists of sheep's cells treated with anti-sheep haemolysin prepared from rabbits. The guinea-pig is the source of complement. The following table illustrates the reaction:

TABLE VII

(Plague bacilli + inactivated specific serum) + complement + haemolytic system ..	no lysis = positive
(Plague bacilli + inactivated normal serum) + complement + haemolytic system ..	lysis = negative

From what has been said above it should be clear that the fixation of complement in a reacting system is a proof of the union of antigen and antibody and that no union will occur unless the antigen and antibody are homologous. Complement-fixation test,

therefore, can be utilised for the identification of a doubtful antigen by using a known immune serum, or as a means of detecting an unknown antibody by using a known antigen. Practically, it is used for the differentiation of bacterial species or in the determination of proteins for medico-legal purposes, when the amount present is too small to be detected by the ordinary methods of precipitation. But more frequently than these, it is employed in the identification of antibodies and extensive clinical application of it is found in the diagnosis of syphilis; when applied in the diagnosis of syphilis it is known as the Wassermann test. Other diseases in which the complement-fixation test is used for diagnosis are yaws, gonorrhoea, tuberculosis and whooping cough; it is also useful in the metazoan infection—hydatid disease.

In the complement-fixation test, if fixation has taken place, the subsequently added haemolytic system remains unaffected and the cells sediment in a few hours. On the other hand, if fixation has not occurred, complement is free and available for the haemolytic action with the result that laking (lysis) follows, leading to the conclusion that either the suspected antibody is absent from the serum under test or the antigen employed is not the one under search. The inhibition of lysis, therefore, signifies a positive reaction and lysis a negative reaction (Table VII). It is important to bear in mind that the test has to be conducted on a strictly quantitative basis. For details refer to the Wassermann test.

No more does it seem necessary to continue the old belief that a special type of antibody is involved in complement-fixation. Any antibody that can sensitise the corresponding antigen may cause the reaction (p. 244). In the Wassermann reaction, after mixing the lipoidal antigen with the syphilitic serum, a drop examined under dark field illumination reveals the occurrence of discernible precipitation. The absorption of syphilitic serum with the lipoidal antigen also removes the flocculating capacity of the former. Further, specific precipitates in a precipitin reaction have the property of fixing complement, even when visible particulation has not taken place. The immune serum employed in the precipitin test also exhibits complement-fixing property. Proof has thus been adduced to indicate that precipitation and complement-fixation depend on a single antigen-antibody reaction. It is probable that the complement-fixing

capacity is most pronounced in the early stage of precipitation when the antigen-antibody complex presents a maximum surface for adsorption.

Other factors that influence complement-fixation are temperature, pH and the turbidity of the antigen emulsion. The order in which the reagents are added may likewise have some significance. An optimum ratio between antigen and antibody is necessary for reaction; excess of either may cause prozone phenomenon.

Non-specific Fixation of Complement: Anticomplementary Reaction. Although antigen or antibody cannot ordinarily fix complement independently of the other, each may separately absorb it in low dilutions. It is then said to be *anticomplementary*. Similarly, several non-specific factors may interfere with the fixation of complement and produce anticomplementary effect. Thus, many tissue cells and the stromata of haemolysed erythrocytes have been found to be complement-fixing without any preliminary sensitisation; so also yeast cells and many bacteria. It is for this reason that in fixation tests, bacterial extracts are to be used in preference to bacterial suspension. Again, normal sera of man and various animals contain natural lysins and exhibit anti-complementary activities; they may also develop such activities by a few days preservation at room temperature but very slowly in the ice chest. The anticomplementary effect is readily destroyed in human serum by heating at 56° C. for thirty minutes, but it is partially stable at this temperature in the case of animal sera.

Haemolytic sera are usually prepared from rabbits by injecting them with graded doses of erythrocytes. The serum collected is mixed with an antiseptic preservative, like phenol or glycerine, titrated and stored in the refrigerator. Under such conditions, it keeps over long periods with little deterioration. High dilution of the haemolytic serum in the actual reaction makes negligible its natural complement which will also be destroyed by prolonged storage and by the antiseptic preservative. In order to make this sure, the sera may be heated at 56° C. for thirty minutes before standardisation.

Titration of Haemolysin. Three variables are involved in this reaction as against two in the precipitation and agglutination phenomena. In titration experiments, the red cells are always kept constant, using a convenient volume, such as 0·5 ml., of a 5 per

cent. suspension. Complete haemolysis in a definite time under specified conditions is taken as the end point. When the haemolysin is to be titrated, complement is also kept constant and *vice versa*. A quantitative relationship exists between the haemolysin and the complement necessary for complete lysis. The amount of haemolysin required for complete action varies inversely with the amount of complement. But this is true only up to a point beyond which, however much we may increase the dose of complement, a certain minimum amount of haemolysin is required to complete the reaction; the opposite also is true. Hence, the minimum haemolytic dose (M.H.D.) of haemolysin or complement is the minimum dose that will cause complete lysis of an arbitrarily selected amount of red cells, in the presence of an excess of the other, in one hour at 37° C. In actual practice, however, complement and haemolysin are usually employed in doses of 3 to 5 M.H.D.

Phagocytosis, Opsonins and Bacteriotropins. Phagocytosis is the ingestion of materials by living cells. Dead or living bacteria, other cells, particles of food or other substances may all be ingested. An example of phagocytosis in general biology is the ingestion of food particles by the amoeba. The devoured element may not always undergo intracellular death and destruction, although that is obviously the purpose of ingestion. Phagocytosis is a general biological attribute of the living cell and in the higher forms of life consititutes an important mechanism of defence against bacterial infection.

The conception of phagocytosis as a possible mode of defence against infection had been foreshadowed by early workers. But it was the fundamental observations of Metchnikoff on the ingestion of bacteria by living cells that laid the foundation of our present knowledge of the subject. From his own observations, Metchnikoff concluded that phagocytosis was the chief factor of the defence mechanism against bacterial invasion and that all other factors were only secondary, and ultimately traceable, to the phagocytes. This, in the light of later work, proved untenable and it is now an established fact that phagocytosis does not occur independently of humoral factors.

Phagocytic Cells. Metchnikoff divided the phagocytic cells of the body into microphages and macrophages, the former represented by the neutrophiles and the latter comprising all the

rest, such as the large mononuclear cells, the wandering tissue cells (clasmatocytes or motile histiocytes) and the fixed tissue cells. From more recent studies, it would appear that the eosinophiles and probably the basophiles are also phagocytic, though not so active as the neutrophiles. Therefore, all the granulocytes are now included under the microphages. The field of the macrophage system has also been further extended since the days of Metchnikoff. A variety of cells distributed throughout the body are apparently potential phagocytes and may be stimulated into phagocytic activity under certain conditions. But certain groups of these have been found to be particularly active and are classed under the modern term of *reticulo-endothelial system*; they include the fixed endothelial cells of the blood and lymph sinuses, mobile histiocytes of the connective tissue, septal cells of the lung, pulp cells of the spleen, the monocytes of the blood and the reticular cells of the spleen and lymphatic glands; the last mentioned cells are the least active. All these are believed to be mesodermal in origin.

The Phenomenon of Phagocytosis. The phenomenon of phagocytosis can be easily demonstrated both by test tube experiments and in the animal body. But more precise information can be obtained by the former than the latter method; in the animal body the conditions governing phagocytosis are far more complicated and far less amenable to control than in the test tube. Leucocytes are the most widely employed cells for in vitro experiments as they are easy to obtain. If suspensions of leucocytes and bacteria are mixed together with some fresh normal serum and incubated for a time, microscopic examination of the mixture will reveal that most of the leucocytes have ingested bacteria. From this numerical values indicating the opsonic capacity of the particular leucocytes can be calculated.

The opsonising capacity of the leucocytes has been found to fluctuate in certain infections and it used to be estimated as a measure of resistance. It is seldom used now. For, the process of intracellular ingestion involves a number of variable factors and, though it can be readily demonstrated, its quantitative estimation is by no means simple nor the results obtained always reliable and accurate.

Many methods have been devised in the study of phagocytosis in the animal body. One of these consists of injecting bacteria

intravenously and estimating the rate at which they disappear from the blood stream. This is done by periodic bleeding after injection and culturing definite quantities of blood by the plate method. This is frequently employed in the study of organisms not readily susceptible to the lytic action of antibodies, such as the staphylococcus, streptococcus and pneumococcus. The results are checked by examining stained sections of spleen, bone marrow, lung and liver for engulfed bacteria. Another method is by injecting the organism into the pleural or peritoneal cavity and collecting the exudate from time to time by aspiration or after killing the animal and examining suitably stained smears for intracellular bacteria. The method of tissue culture has also been pressed into service for investigating the phagocytic property of different types of tissue cells. But it should be remembered that the behaviour of cells in tissue culture may not be strictly analogous to what is obtained in the animal body.

Opsonic Index. A standard suspension of the test organism is mixed with equal quantities of normal serum and a standard suspension of washed leucocytes. The mixture is aspirated into capillary tubes and incubated at 37° C. for fifteen minutes. Thus, a number of normal sera are separately put up. After incubation, the contents of all the capillary tubes are pooled together, smears are made from it, stained appropriately and examined. Organisms are found to have been ingested by the neutrophiles in varying numbers. The number of intracellular bacteria is counted in an arbitrary number of leucocytes, usually 50–100; the average number of organisms per leucocyte is called the *phagocytic index*. The same procedure is repeated but substituting the patient's for the normal serum. The ratio of the phagocytic index of the test serum to that of the normal serum is called the *opsonic index*.

Great care should be bestowed upon the technique of the test. Incomplete mixing of the reagents will prevent the meeting of the leucocytes and bacteria. A uniform distribution of the leucocytes is particularly important. Prozone phenomenon may sometimes develop; hence, it is necessary to put up a series of dilutions. It is to be remembered that a young culture resists ingestion more than an old one. Again, stock cultures are unsuitable as they are likely to be in the dissociative phase when they are more vulnerable than when in the smooth virulent phase.

In working out the opsonic index of patients, some workers prefer to use the patient's leucocytes and the infecting strain as the organism. All the above mentioned sources of inaccuracies render the conduct of the test difficult and even when great care is taken the experimental error is very high. That is why the test is not of much practical value.

The complex phenomenon of phagocytosis has been extensively studied and some of the fundamental facts connected with it have been brought to light. It was Metchnikoff who first showed that the presence of bacteria in a cell was not due to an invasion of the latter by the former, as was then believed, but was the result of the cell actively ingesting the bacteria. Cells exhibiting such devouring capacity he named *phagocytes*. At first he believed that phagocytosis was purely a cellular process, but soon he discovered that the body fluids contained certain substances that promoted phagocytosis and that in the absence of these leucocytes failed to ingest bacteria. Leucocytes washed free from all traces of serum were thereby practically deprived of their power of phagocytosis. However, the addition of a small quantity of fresh normal serum was found to restore the lost property. From these observations Metchnikoff formulated that certain factors present in the blood were necessary for phagocytosis but that they acted through the leucocytes and by stimulating them.

The exact role of serum in relation to phagocytosis was not, however, clear. Wright and Douglas confirmed the previous observations that certain serum factors were essential for phagocytosis and that without their intervention, ingestion of bacteria did not occur. Prior to them, the possibility that the action of serum might be on bacteria had been suggested by others. But it was Wright and co-workers that demonstrated conclusively by adequate experimental data that the phagocytosis-promoting factor in the normal serum acted not on the phagocytes, as propounded by Metchnikoff, but directly on the bacteria, rendering them readily ingestible. Washed leucocytic suspension, when mixed with a culture of staphylococcus and incubated, was found to be powerless to ingest the cocci. But the addition of a small quantity of fresh serum was found to enable the leucocytes to engulf the organism. This proved the dependence of leucocytes on serum for their phagocytic function. The staphylococcus

was then mixed with serum and incubated. After washing the organism well with isotonic salt solution to get rid of all traces of serum, it was mixed with washed leucocytic suspension and incubated. Ingestion of the organism took place without any further addition of serum. From this they concluded that the serum factor acted on the bacteria, preparing them for ingestion. In order to express this, they named the serum factor *opsonin*, meaning I prepare food. Thus, the contention of Metchnikoff that the serum factor acted through leucocytes was proved erroneous.

Bacteriotropins. At the same time Neufeld and Rimpau showed that active immunisation increased the opsonic activity of serum and named the responsible factor contained in the immune serum *bacteriotropins*. Due to the functional similarity with the normal opsonin, it is also referred to as the immune opsonin. In fact, some workers consider that there is very little difference between the natural opsonins and bacteriotropins and they use these terms interchangeably. Bacteriotropin is far more active than the natural opsonin. Unlike the normal opsonin, bacteriotropins are relatively thermostable and immune serum when heated to a temperature of 56°–60° C. for thirty minutes does not lose its opsonic activity. Likewise, it differs from the normal opsonin in that it is specific, acting only against the particular organism employed for immunisation and not against others. Bacteriotropin appears to be analogous in its action to precipitin and agglutinin, sensitising the bacteria, probably by forming a surface coating on the bacterial cell and reducing the surface tension, as a preparatory step to phagocytosis.

Opsonins. Opsonins are normal constituents of blood which sensitise bacteria and render them vulnerable to phagocytosis. Their exact mode of action is not known; probably they form a surface deposit on the bacterial cell and lower its surface tension, rendering it easily ingestible. They are heat labile being destroyed at 56°–60° C. in ten minutes. At ordinary temperature, the opsonic activity of normal serum rapidly declines. Exposure to light likewise results in the extinction of this property. Opsonins are relatively non-specific, acting on a variety of organisms. The opsonic power of a normal serum varies widely towards different species of bacteria; conversely, the same bacterial species exhibits wide variations in its susceptibility to normal sera derived from different individuals.

The exact nature of opsonin is uncertain. Some identify it with the complement, while others maintain that it is a distinct entity. While there are certain similarities between them, like non-specificity and lability, there are also points of dissimilarity. The elimination of complement action does not completely deplete a serum of all its opsonic potency. On the other hand, a selective absorption of opsonin is possible from fresh normal serum, at least in some cases, without interfering with its haemolytic property. Again, dilute ammonia deprives normal serum of its complement activity but does not abolish its opsonic power. There is also suggestive evidence that the opsonising power of normal serum may be due to the presence of two factors, a thermostable substance resembling tropins and a thermolabile substance resembling complement. While the first can act by itself, the second cannot, though it does enhance markedly the action of the other.

Other Influencing Factors. Certain chemical and physical factors influence phagocytosis. The optimum temperature for phagocytosis is the normal temperature of the species concerned; for man it is 37° C. Irradiation with ultraviolet rays or X rays does not seem to improve appreciably the activity of the phagocytic system of the body, as was once believed. If done in excess, it has a depressant effect both on the phagocytes as well as on the serum factor. A preliminary exposure of bacteria, on the other hand, makes them more vulnerable. Certain ions exert a marked influence on the ingestive capacity of phagocytes. For example, citrate ions have a depressing effect and calcium a restoring effect. The use of citrated blood is, therefore, contra-indicated in determining the opsonic index. The pH is another important factor. The optimum activity occurs at about neutrality, or slightly on the acid side; any departure from it has an inhibitory effect. It may be that any such deviation will injure the phagocytes or interfere with the action of opsonins. The hydrogen-ion concentration of the environment may also play a role in determining the type of cells migrating to an inflammatory focus. Isotonicity of the medium is another necessary criterion. Phage degeneration of bacteria seems to render them more susceptible to phagocytosis. Bacteriophage is also said to exercise a stimulating influence on phagocytes.

Marked fluctuations in the ingestive capacity of leucocytes are encountered in health and disease. The phagocytic power

of leucocytes is also not so marked at birth as in the adult. It is said that it does not reach its maximum until about the age of three. The age of the leucocyte itself may not have any significant influence on its phagocytic power.

The susceptibility of a bacterial species is profoundly influenced by its virulence and antigenic structure. The smooth virulent forms are not so readily ingested as the rough avirulent ones. The capsulated organisms display a high degree of resistance to phagocytosis; in their non-capsulated state they are more vulnerable. In the case of the pneumococcus, for example, the smooth capsulated forms are highly resistant but not so the stock culture. Of considerable interest is the observation that the presence of the capsular substance markedly inhibits phagocytosis even in the presence of the immune serum. Again, older cultures and stock cultures are readily susceptible. It may be reminded that in all these cases the surface antigens are considerably altered, being replaced by less virulent or non-virulent antigens. The increased vulnerability of phage-affected bacteria is also due to the same cause.

The Fate of Ingested Bacteria. The ingested organisms are not invariably destroyed. Where destruction occurs, and it is so in a large number of cases, the organisms show swelling, granulation and fragmentation in succession before the final intracellular disappearance. This digestion of ingested bacteria does not appear to be a process of intracellular lysis brought about by an immune lysin and complement as was originally suggested.

The neutrophile leucocytes appear to be responsible for dealing with organisms in localised infections and probably also for clearing the blood stream of the attacking bacteria. They are the first to appear at any site of inflammation and so constitute the first line of defence. They display great activity and rapidly engulf large numbers of the invading bacteria. Ingestion may occur whether the bacteria are dead or alive. When they are alive, ingestion may or may not result in their ultimate destruction. In many cases, it does not; for example, ingested tubercle bacilli are not destroyed; even organisms like the staphylococcus and gonococcus, which are more vulnerable, remain viable within the cell over long periods and may even multiply. The gonococcus has been shown to grow in the

ingested cell. What is said regarding the fate of the ingested organisms in the leucocyte is also true of those ingested by the macrophages. For instance, engulfed lepra bacilli, rickettsia and some of the viruses remain apparently alive and even grow. Indeed, the possibility is there that in all cases, where death does not supervene, such bacteria-laden cells, when motile, may carry infection to other parts of the body and thereby prove harmful. In their intracellular position these bacteria are protected from the bactericidal and lytic factors of exudates and plasma.

When final destruction is the sequela of phagocytosis, death of the organism is a necessary prelude to it. How this killing is brought about, is not quite clear. It is a well known fact that leucocytes contain a variety of bactericidal ferments. The term *leukins* has been applied to some of these; they are probably responsible for the death of the ingested parasite. They may also occur in normal blood and contribute to its bactericidal effect. It is more than likely that leukins exert a selective action on different species of bacteria. Some bacteria are readily killed, while others are not equally susceptible to their action. How exactly dissolution occurs after death, is also a mystery. Probably, other intracellular enzymes may have a hand in it. Extracts capable of splitting proteins, carbohydrates and lipoids have been obtained from cells found in exudates.

It was originally thought that microphages were the only active cells against bacterial invaders and that macrophages formed the scavenging system concerned with the disposal of dead tissue and debris. It has been indisputably proved both by in vivo and in vitro experiments that not only the granulocytes but also the macrophage cells are actively phagocytic towards bacteria sensitised by normal or immune opsonin. In fact, there is overwhelming evidence indicating that the macrophages of the reticuloendothelial system play a dominant role in dealing with the invading parasites and in determining recovery from infection.

The Mechanism of Phagocytosis: Chemotaxis. The mechanism of phagocytosis is a complex one involving several factors concerning the phagocytic cells, the ingestible particles and the environment of ingestion. How the meeting of the phagocyte with the parasite is brought about and by what mechanism the former ingests the latter, are two of the central problems of which our knowledge is still incomplete. That contact between

the phagocyte and the invading parasite is an essential prerequisite for ingestion, is self-evident. How this contact is established, has been the subject of much experimental study and as the result of it the mechanism of *chemotaxis*, or chemical attraction, has been postulated as the agency through which contact is brought about.

Infection starts an inflammatory reaction at the point of invasion of an organism, characterised by increased capillary permeability, allowing the escape of leucocytes and fluids into the surrounding tissue spaces. Here the leucocytes move about in virtue of their natural amoeboid movements. But the meeting of the cells with the bacteria is not a chance occurrence. During infection some of the invading bacteria and some host cells die. Certain chemical substances emanating from the dead and living bacteria and disintegrating cells diffuse into the surrounding area, and on reaching the phagocytic cells bring about alterations in the surface forces of these cells; such alterations tend to direct the flow of their protoplasm towards the point of origin of these chemical substances, that is, in the direction of the bacteria. This is what is called *positive chemotaxis*. The contact of phagocytes with the organism is, therefore, the result of a selective movement conditioned by the environmental factors of the phagocytic cells. Positive chemotaxis appears to be a general attribute of all pathogenic organisms. It is true that all the components of chemotaxis have not been understood, but there is no doubt that the chemical directive provided by the invading parasite is responsible, partly at least, for the attraction of the leucocytes towards the point of infection. It is interesting to note that recently a nitrogenous substance, which enhances the capillary permeability and exerts positive chemotaxis, has been isolated from sterile inflammatory exudates; it has been named *leukotaxine*. Such products as this, formed as the result of tissue injury, may explain the migration of leucocytes to foci of non-bacterial inflammation. There is also evidence suggesting that even the disintegration of leucocytes might provide the chemotactic stimulus.

The actual ingestion is also not a simple process. Having come to the neighbourhood of the foreign element and established contact with it, the phagocytic cell is again subject to the stimulus of similar forces which cause changes in its surface

tension at the point of contact, enabling it to engulf the foreign particle. Viscosity of the protoplasm of the ingesting cell and temperature are two other important influencing factors in the process of ingestion. On the other side are the bacteria prepared for ingestion. The immune opsonins, or in a less degree the normal opsonins, have sensitised the bacterial cell probably by adsorbing on the surface, lowering its surface tension and rendering it fit for ready ingestion.

The foregoing hypothesis has not been accepted by all; it may not completely explain the mechanism of positive chemotaxis. There may be still other factors involved in this process, which at the moment we are unaware of. It is also highly probable that other factors, besides what have been already alluded to, are also vitally concerned with the whole phenomenon of phagocytosis. It is significant that an increased oxygen consumption has been observed to accompany phagocytic activity.

CHAPTER XIII

HYPERSENSITIVENESS

Hypersensitiveness is an abnormal state of an individual, animal or man, characterised by an increased specific reaction capacity to a substance which produces little or no reaction in normal individuals of the same species. For example, the ingestion of egg white does not usually produce any untoward effect in man. But in a very small percentage of people it may produce violent gastro-intestinal symptoms, severe urticaria, eczema or asthma every time they take egg. Hypersensitiveness is produced in a variety of ways; and in certain types, represented by protein anaphylaxis, the production of antibodies and their participation form the essential part of the anaphylactic phenomenon. In others no such changes are definitely traceable; but, because of the similarity of features, the operation of a similar machanism has been regarded as possible even in these reactions.

Of all the specific changes occurring in living tissues from the impact of foreign antigenic substances, hypersensitiveness is the most delicate, expressing itself in a more delicate manner than all other conditions. The hypersensitive state, unlike immunity, reveals itself by an exaggerated reaction associated with characteristic symptoms, often of a spectacular nature.

The term hypersensitiveness is employed in a general sense to embrace all types of exaggerated reactions, whether occurring naturally in man or induced artificially in animals. The term *anaphylaxis* is used to denote the artificial condition of hypersensitiveness induced in lower animals, in which an antigen-antibody mechanism is clearly demonstrable. The term *allergy*, originally introduced by Von Pirquet to denote hypersensitiveness in general, is reserved to cover all hypersensitive manifestations occurring in man. *Idiosyncracy* and *atopy* are other terms used in human hypersensitiveness. The former is sometimes used in drug hypersensitiveness and the latter to indicate those hypersensitive conditions in man which are definitely influenced by

genetic factors. There appears to be scant justification for this differentiation. The antigen responsible for the anaphylactic type of hypersusceptibility is called *anaphylactogen*; *anaphylactin* is the term applied to the corresponding antibody; *allergen* (atopen) and *allergin* (reagin) are the corresponding terms in allergy.

Anaphylaxis

The systematic study of the subject of anaphylaxis dates from 1898 when Richet and Hericourt, during their attempts to immunise dogs with toxins, found that these animals treated with eel serum, which is toxic in itself, were killed by a second dose of the same substance, too small to cause any serious reaction in normal untreated animals. Later Richet and Portier made similar observations on the same animal with a glycerine extract of the tentacles of *Actinaria*, also a toxic substance. They found that dogs, which had been previously injected with minute doses of the extract, reacted violently and often died when bigger, but still sublethal, doses of the same material were injected intravenously. Thus, instead of causing protection, they found that the initial injection produced the opposite effect. In order to express its antithesis to prophylaxis, they called (1902) this phenomenon *anaphylaxis*, meaning without protection.

Their observations opened up a new field of research and the problem was studied independently by many workers. But it was Otto's experiments on guinea-pigs with horse serum, a non-toxic substance, that formed the basis of our present knowledge of the fundamental facts of protein sensitisation. The phenomenon was later studied in various animal species, employing a variety of antigens, further widening the field of knowledge. These investigations have also proved invaluable, as most of what we know about human allergy is based on observations made on anaphylaxis induced in lower animals.

Active Sensitisation. In order to produce anaphylactic sensitiveness to a substance in a susceptible animal, a minute dose of it, called the *sensitising dose*, is injected into the animal. After a variable interval, called the *incubation period*, the animal develops hypersensitivity; it is then said to be *anaphylactic*. This can be demonstrated by injecting a second bigger dose of the same substance intravenously when the animal develops a characteristic acute symptom complex, termed *anaphylactic shock*, and

often succumbs to it. The second dose is variously known as the *toxic*, *shock*, *exciting* or *assault dose*. For example, 0·1 c.c. of horse serum is injected into a guinea-pig. Nothing happens to the animal immediately, for horse serum is not of itself toxic to these animals. After a period of fourteen days, a second dose of 1 c.c. is given intravenously. In a few seconds or minutes the animal develops acute shock and dies.

The anaphylactic state can also be demonstrated by the Schultz-Dale Experiment. Strips of intestines or uterus from the sensitised guinea-pig is suspended in oxygenated Ringer's solution and the specific antigen, horse serum, is added to the bath. The muscle strip undergoes spasmodic contraction, similar to that produced by histamine, which can be recorded with a kymograph. The tissue does not respond on a second addition of horse serum, because it is desensitised. Strips from a non-sensitised animal do not exhibit any contraction.

The Antigen. All substances are not capable of acting as anaphylactic antigens. Only those which have the same nature as antigens in general are able to produce the anaphylactic state *i.e.*, they should be foreign proteins capable of inciting antibodies (p. 231). The purer the protein, the smaller the dose required to sensitise and the greater the thoroughness with which sensitisation can be effected. Foreign sera and egg albumin are examples of efficient anaphylactogens. The proteins of bacteria and protozoa can also function as anaphylactic antigens. The sensitising property also depends upon the physical state of the antigen; it should be such as to diffuse readily into the body fluids and quickly come in contact with the tissue cells. Denaturing of antigens by heat, chemicals or by other methods results in the loss of the sensitising quality.

As in immunisation, the same animal can be rendered sensitive at one and the same time to a variety of antigens, reacting specifically to each on subsequent injection. There may not be uniformly intense reactions to the several antigens.

Partial antigens, or haptens, will not sensitise but may, nevertheless, induce shock in an anaphylactic animal. This, however, is possible only with complex haptens, like the bacterial polysaccharides. Simple haptens are unable even to induce shock; but when injected into the anaphylactic animal, they inhibit the production of shock by the full antigen. Lipoids and

carbohydrates can be transformed into anaphylactogens by conjugation with proteins, and studies with such synthetic proteins have thrown a flood of light on many problems connected with human hypersensitiveness.

Specificity. In all anaphylactic phenomena, as in the case of immunity reactions in general, a high degree of specificity relations prevail. If, for example, horse serum is employed for sensitisation, no other antigen is capable of inducing shock except horse serum or an antigenically related substance. In close analogy with immune antigens, specificity depends upon the chemical structure of the anaphylactogen and not upon its biological source. But, as the same biological species practically always exhibit identical chemical make-up, chemical and biological specificities largely overlap.

Sensitising Dose. As a rule, the sensitising dose should be very small, only just enough to incite antibody formation and not such as to cause the presence of a large concentration of antibodies in the blood. In fact, the success of the reaction depends upon the absence of an appreciable amount of circulating antibodies; if they are present in adequate amounts, they will engage the antigen before it can reach the cells and thereby prevent the production of shock. The quantity of antigen required to sensitise also depends upon the nature of the antigen, the animal species and the route of introduction. Very small doses given by the intravenous route are enough to sensitise, whereas by any other route much larger doses are necessary to produce the same effect.

All animals are not prone to active or passive sensitisation. Guinea-pigs, rabbits and dogs can be readily rendered anaphylactic. Cats, rats and mice are difficult to sensitise, while monkeys are extremely refractory. The symptoms of anaphylactic shock vary markedly in different species but are remarkably identical in members of the same species, irrespective of the type of antigen employed. For example, horse serum produces in all guinea-pigs the same symptom complex and pathology; so also egg albumin or any other antigen. But the symptoms produced by horse serum or egg white in rabbits are quite different. Notwithstanding these variations, the underlying mechanism appears to be identical in all the species, and the divergencies can be satisfactorily explained by the peculiar histology of each species.

Incubation Period. As in the case of active immunisation, an incubation or latent period is necessary before anaphylaxis can be fully established. The duration of this period depends upon the amount of antigen, the route of injection and the type of the animal. Large quantities of antigen may prolong the incubation period, probably by remaining longer in the blood and causing the neutralisation of the antibodies as soon as they are formed. But within limits, the smaller the dose, the longer the incubation period, as it takes longer to generate sufficient antibodies. The latent period is slightly shorter after the intravenous route. Short as it is, it takes some time for the antigen to be absorbed into the circulation after the subcutaneous route. The incubation period is also shorter for guinea-pigs than for dogs and rabbits.

Duration. Once sensitised, the animal remains so during the rest of its life. Hypersensitivity may wane in course of time but never disappears entirely. It fades less slowly in guinea-pigs than in dogs and rabbits.

Passive Anaphylaxis. As in immunity, the anaphylactic state can also be induced passively by the injection of the serum from an actively sensitised animal; it can also be done with immune serum. The animal in this case is sensitised not as the result of any antigenic stimulus but in virtue of the antibodies transferred with the sensitising serum; its tissues themselves have no part in the production of the sensitising antibody and the condition is created passively. However, after the sensitising dose a definite period should elapse before the hypersusceptible state is established and during this period the antibodies would have practically disappeared from the blood. This has been taken to be a proof that the essential anaphylactic reaction is a cellular phenomenon and that the latent period is the time required for the cells to fix the circulating antibody. The latent period may vary from 24–48 hours, but it may be as low as four hours if the sensitising serum is given intravenously. The shock dose should, of course, consist of the same antigen as employed in the active sensitisation, or immunisation, of the animal wherefrom the sensitising serum for passive immunisation is taken. Sensitisation passively established disappears in three to four weeks. The offspring of an anaphylactic mother may also react positively for a short period after birth as the result of passive transfer of sensitiveness.

Nature of Antibody. It is not doubted that anaphylaxis depends upon the presence of antibodies. But their exact nature is not clear. Some regard that precipitin is the antibody concerned. There is some evidence in support of it. Precipitating sera are usually sensitising and shock producing. But this evidence is weakened by the fact that sensitisation can be transferred in the absence of demonstrable precipitins in the plasma. To counter this it has been argued that its concentration in the plasma may be too minute to be detected by the ordinary precipitation phenomenon and that its presence can be revealed only by the anaphylactic reaction which, as we know now, is far more delicate than the former. It is also pointed out that only minute doses are enough to cause sensitisation. Further, the absence of precipitin from the plasma in such cases is also explained by its cell-fixed position. The problem is still not completely solved, but there is no reason to believe that the anaphylactic antibodies are different from immune antibodies. The question whether they are identical or not with precipitins loses its importance if the unitarian conception of antibodies is accepted (p. 243).

Inheritance. The anaphylactic state may be inherited. Intrauterine sensitisation may occur in two ways: actively by antigen brought to the offspring through the maternal blood and passively by the passage of antibodies from the same source. Children of an anaphylactic mother may react positively for a short period after birth, about 8–10 weeks.

Shock Dose. The shock dose should in all cases be considerably bigger than the sensitising dose. The best results are obtained by giving it directly into the circulation, as that would bring the injected antigen suddenly into contact with the sensitised tissues. Administered by any other method, absorption is slow and contact of the excitant with the sensitised cells is gradual, lessening the intensity of the reaction. Considerably larger amounts may, however, compel speedy absorption of the optimum dose necessary for the production of explosive reaction.

Symptoms of Anaphylactic Shock. Anaphylactic shock varies widely in severity. Acute reaction is most readily elicited in guinea-pigs sensitised with horse serum. In a short period, ranging from a few seconds to a few minutes after the assault dose, the animal develops a characteristic train of symptoms, leading to death in severe cases. The animal at first becomes

restless, its fur, particularly over the head and neck, becomes ruffled, it rises on its hind legs, begins to sneeze and cough, and paws its face. It may jump. Increased respiration is noticeable. In mild cases the respiratory difficulty passes off, and the animal gradually recovers in the course of several hours. In severe cases the animal falls on its side. Muscular twitchings develop. Respiration becomes slow and shallow; the dyspnea is inspiratory. Convulsions set in, the animal tries to sit up and run but falls down and breathing stops.

Shock is accompanied by a number of secondary changes. There is a fall of blood pressure preceded by a temporary rise due to a preliminary vasoconstriction. There is also a fall of temperature, fall in the complement content of blood, lowered coagulability of blood, temporary leucopenia and eosinophilia. The post-mortem appearances are likewise characteristic. Hyperinflation of the lungs is a marked feature due to the confinement of air in the alveoli by the severely constricted bronchial tubes; they are pale and bloodless and do not collapse after opening into the pleura. Heart continues to beat for some time after breathing has stopped. Death is due to asphyxia.

Rabbits cannot be so easily sensitised as guinea-pigs. Several doses have to be given before complete anaphylaxis can be established in them. On induction of shock, death may follow in a few seconds or few hours. It is due to acute heart failure and not due to suffocation as in the guinea-pig. On post mortem, instead of the extreme pulmonary distension found in guinea-pigs, there is severe dilatation of the right side of the heart from extreme narrowing of the pulmonary arteries. Respiratory movements may continue for a short period after the heart has stopped beating.

It is not easy to sensitise dogs and they also require several sensitising doses to create complete sensitisation. The liver is very much enlarged and congested due to narrowing and obstruction of the hepatic vein. Death is probably due to the fall of blood pressure consequent on splanchnic engorgement.

Although the general symptomatology of anaphylactic shock varies in different species of animals, there are, however, certain basic features common to all species. Such are the vasomotor changes consisting of a temporary initial constriction followed by dilatation and fall of blood pressure, general irritability and

spasmodic contraction of smooth muscles, marked drop in the coagulability of blood due to a fall in its fibrinogen content and leucopenia. Although oedema is a common feature in practically all local manifestations of hypersensitiveness, as in the various cutaneous and other reactions, it is not so in anaphylactic shock.

It has been mentioned above that variation in symptoms and their underlying pathology observed in the three species of animals can be explained by the differences in their anatomical and histological structures. In anaphylactic shock the smooth muscles of the body undergo spasmodic contraction. They are particularly concentrated in the bronchial tubes of the guinea-pigs and their tetanic contraction eventually leads to pulmonary emphysema and asphyxia. In contrast to it, the bronchial musculatures of rabbits and dogs are poorly developed. On the other hand, the musculature of the pulmonary artery of the rabbit is well developed and the extreme contraction of it leads to distension and failure of the right side of the heart, resulting in death. In the dog the hepatic vein is found to be considerably narrowed and the resulting obstruction is apparently the cause of acute congestion and enlargement of the liver, leading to a depression of the systemic blood pressure, too severe to be consistent with life. In this animal the smooth muscles of the hepatic vein are found to be well developed.

The available evidence indicates that the contraction of the smooth muscles is the result of a local and direct stimulation by some substance. If the nerve supply of the bronchial muscles is cut off or blocked, anaphylactic shock can still be induced.

Desensitisation and Anti-Anaphylaxis. These terms are often used interchangeably. Strictly speaking, the term desensitisation should be applied for any attempt to abolish an anaphylactic state already established and anti-anaphylaxis should be reserved for any interference with the process of sensitisation.

Provided the second dose is given after the completion of the sensitising process, a characteristic anaphylactic shock, usually ending fatally, can be readily elicited. If the second dose is given three days after the initial dose, it will only prolong the incubation period, but when given towards the late stage of the incubation period, that is 8–10 days after the first injection, the animal, as a rule, recovers following reactions of varying severity. In such

recovered animals it is not possible afterwards to set up anaphylaxis against the same antigen. They are said to be refractory or anti-anaphylactic.

In order to desensitise an anaphylactic animal, a series of sublethal or fractional doses of the specific antigen should be administered over long periods, avoiding at any time too big a dose to cause explosive reaction. The desensitising doses may be given by any route, but the intravenous or the intracerebral route produces the effect most promptly. The process of desensitisation is really one of causing a series of shocks, too trivial to cause much damage to the sensitised cells. Desensitisation is attained probably through a process of fractional neutralisation of the cell-fixed antibody every time the specific antigen is injected, thereby causing the ultimate depletion of the former from the sensitised cells. The refractoriness so produced is, however, only temporary, lasting for a few weeks. A probable explanation of this is that the desensitising doses may, besides neutralising the antibody, also act as fresh stimuli for further sensitisation.

Local Anaphylaxis or Arthus Phenomenon. This is a curious phenomenon observed in rabbits. When horse serum is repeatedly injected subcutaneously into them, at first little or no reaction is provoked. But after a few injections, when the animal becomes increasingly immune, local reaction consisting of swelling and oedema begins to appear, increasing in intensity with every successive dose and finally giving rise to marked induration and necrosis. The reaction is not necessarily confined to the site of the earlier injection or its immediate vicinity but can be elicited at any site. It can also be provoked on rabbits previously immunised with horse serum and showing a high content of the specific antibodies. The reaction is unaccompanied by any general disturbance. This is an example of local anaphylaxis and is called the *Arthus phenomenon*, as he was the first to observe it. Attempts to induce it in guinea-pigs and dogs have not succeeded. Desensitisation fails to abolish the local reaction and the difficulty of desensitising cutaneous allergy in man is probably a reflection of it. A human counterpart of the Arthus phenomenon is sometimes found at the site of serum therapy or antirabic injection; indurated areas develop at the site after a few injections. The phenomenon of Arthus has been invoked to explain the acute manifestations of rheumatic disorders.

Reversed anaphylaxis is the name applied to an interesting local reaction. It is elicited as follows: horse serum is first injected into the blood of a normal rabbit; twenty-four hours later anti-horse serum is injected subcutaneously, when an inflammatory oedema develops at the site of injection. A control injection of normal horse serum does not cause any reaction.

Anaphylactoid Reactions. Symptoms closely simulating anaphylactic shock are produced by the primary injection of various protein as well as non-protein substances, such as toxified serum, serum of certain animals, heterophile antiserum, peptone, histamine, arsenobenzene compounds, morphine, certain bacterial vaccines and others. Any of these substances injected intravenously into the guinea-pig will give rise to symptoms with often striking similarity to those of anaphylactic shock. But there are also certain differences; for example, the characteristic pulmonary changes of anaphylactic shock are absent. The term *anaphylactoid reactions* has been applied to them in order to express their close clinical similarity with true anaphylactic reactions. Administration by the intravenous route is the best way of eliciting reaction. Phenomena sometimes referred to as nitritoid crises and haemoclastic crises are also included in this category. In none of these cases are the reactions referable to any specific antigen-antibody mechanism. It is to be noted that some of these substances are non-protein in nature and are unable to produce either active or passive sensitisation or desensitisation. Colloidal nature is a property common to most of them. Of all substances, peptone and histamine evoke symptoms almost identical to those of anaphylactic shock. It is interesting and suggestive of some common factor in the production of anaphylactoid and anaphylactic reactions.

Mechanism of Anaphylactic Reaction. The specific interaction between antigen and antibody is believed to be the essential factor in the genesis of shock. Where this union occurs, what the immediate biological changes are and why it leads to the production of shock instead of neutralisation of the toxin and protection, are all matters which await final decision.

The exact site, where the antigen-antibody reactions occur, is a matter of dispute. One view is that the anaphylactic reaction is a cellular phenomenon in which the antigen and antibody meet in or on the tissue cells and unite there. That a latent period is necessary to establish passive anaphylaxis after the sensitising

dose, seems to support this view. For, if the blood stream were the site of union, one would expect the establishment of the hypersensitive state immediately the sensitising dose is injected when the concentration of antibody in the blood is the highest and not after the antibody has practically disappeared from the blood. Schultz-Dale experiments with the sensitised uterus, free from all blood or tissue fluids, provide further proof of the tissue location of the antigen-antibody union. This is the cellular theory. An alternate theory is the humoral theory. According to this, the union of antigen and antibody takes place in the blood stream or in the tissue fluids and a special toxin, called *anaphylotoxin*, is formed from the disintegration of the antigen-antibody complex, probably under the influence of complement or some other factor. It is toxic and is responsible for the production of crisis. There is very little evidence in support of this theory and much against it. The immediate response of the isolated uterine strip tends to disprove any extra-cellular position for the combination of antigen and antibody. Further, it has been shown that the sensitive state persists and shock can be induced even after sensitised animals are exsanguinated and reinfused with blood from non-sensitised normal animals. This also does not harmonise with the humoral theory.

The exact group of cells concerned with the anaphylactic phenomena has been suggested to be some component of the reticulo-endothelial system. There is some evidence indicating that the endothelial cells of the capillaries play a part in the mechanism of anaphylaxis; there may be yet others. Wherever the initial reaction occurs, the final brunt of it seems to fall on the smooth musculature of the body; the primary union need not occur in this tissue.

Different views have been advanced about the outcome of antigen-antibody union believed to be occurring in or on the cell. One is that the interaction leads to a profound disturbance in the colloidal state of the cell and that the shock is the clinical expression of this disturbance. Another view is that the interaction inflicts severe injury to the sensitised cells, initiating certain chemical changes; as a result the injured cells release histamine or a histamine-like substance which, acting upon certain susceptible tissues, leads to the production of symptoms. The amount of histamine liberated depends upon the extent of tissue damage

and determines the intensity of the shock. Thus, shock is a secondary histamine effect, a manifestation of auto-intoxication.

Histamine has powerful irritative properties on smooth muscle and on injection into animals reproduces faithfully the symptoms of anaphylactic shock. It is a decarbolised product of histidine. The latter is a constituent of all complete proteins and has been shown to be present normally in the various tissues of the body in varying concentrations.

More recently, it has been shown that the blood of dogs and guinea-pigs removed during anaphylactic reactions exhibited histamine activity. Further, the tissues of anaphylactic guinea-pigs were found to release histamine on the addition of the specific antigen to the perfusion fluid. These, if corroborated, afford direct proof of the liberation of histamine during shock, at least under experimental conditions.

The immediate cause of anaphylactoid reactions may be the same as that of anaphylactic reactions and the symptoms of the former may also be due to the action of histamine. If so, it is not clear what constitutes the cause of the primary cell injury as a result of which the liberation of the shock-producing substance occurs. It is not any antigen-antibody interaction; the underlying mechanism remains obscure.

Anaphylaxis is allied to immunity. In both there is the production of antibodies and their participation in the respective reactions in a specific manner. In anaphylaxis most of the antibodies are deviated from the blood stream to be fixed by certain tissue cells, but in immunity they remain in circulation and block the antigen from reaching the sensitised cells. At this point the divergency between the two conditions begins. The crux of the problem is, if the above theory is accepted, why such a disappearance of the antibodies should take place. It still remains a mystery. The theory has been advanced that whereas the basis of immunity is the antibody globulin produced by the cells and sent into circulation, that of anaphylaxis is the antibody protein produced by the cell but retained within it. Whether the antibodies are not deviated, as mentioned above, but produced and retained within those cells and whether they possess any specific affinity for those cells or *vice versa*, are all problems which remain at present at the level of speculation. From a practical point,

however, the anaphylactic individual harbours a potential danger, whereas the immunised one a protective weapon.

Anaphylaxis in Man. Obviously, it is not possible to conduct the risky anaphylactic experiments on human beings. The type of acute anaphylactic shock induced in animals has not been observed to occur spontaneously in man, except very rarely as the result of serum injections. But such reactions do not have all the criteria of a true anaphylactic crisis, and it is doubtful whether the latter term can be applied to them.

Hypersensitiveness in Man or Allergy

Allergy is an exaggerated reaction capacity occurring spontaneously in man and resembling, in a general way, the anaphylactic state experimentally induced in lower animals. The results of investigations on the latter have shed considerable light on the former's pathogenesis; and there is a good deal of evidence pointing to a common basis for these two types of hypersensitiveness.

Hypersensitiveness in man takes on a variety of clinical forms; there are many factors common to them all. Like anaphylaxis, allergy can be induced both actively and often passively. The reaction is specific in both conditions. In many cases of human hypersensitivity an antigen-antibody relationship can be demonstrated. Further, there is much similarity in the clinical manifestations of the two conditions. But there are also certain dissimilarities between them, making it necessary to deal with human hypersensitivity separately from the experimental anaphylaxis. Our knowledge about these conditions is still imperfect and as it becomes more and more complete, these differences may also be expected to vanish. It has been reported that such allergic phenomena like sneezing, asthma and eczema may naturally occur in lower animals; it is important to pursue this line of search and extend our knowledge before we seek to establish any causal identity between these and similar conditions occurring in man.

In contrast to the artificially induced anaphylaxis, sensitisation in allergy is a natural process. There are also infinite possibilities of sensitisation in human beings, both in utero and after birth. Not only proteins but also a variety of non-protein substances are found to act as allergens and evoke allergic response. Human

hypersensitivity is subject to genetic influences, which anaphylaxis is not. Anaphylactic reaction is usually a general reaction, but allergic manifestations tend to be localised. While anaphylactic symptoms are usually referable to the same organs in all members of the same species, this constancy does not exist in the case of human hypersensitiveness. The organs involved in the latter vary in different individuals; even in the same individual different sites—respiratory, gastro-intestinal and cutaneous—are affected by the same antigen. True, this is mainly due to the fact that spontaneous sensitisation may occur through any of these routes. Again, the immediate cause of the production of symptoms is very often oedema of the parts and not spasm of the smooth muscles. Further more, desensitisation in allergy is far more difficult to achieve than in anaphylaxis. All the above divergencies may be more apparent than real and many of them can be explained without difficulty.

Heredity and Human Hypersensitiveness. It is now all but established that hereditary factors (transmitted through chromosomes) play an important part in human hypersensitiveness. Man is beset with unlimited opportunities for sensitisation, and yet only a small proportion develops sensitiveness. This, undoubtedly, is due to the operation of hereditary influences in these few and the absence of it in the rest. Such influences tend to run in families, but parents and offspring may not exhibit hypersensitivity to the same substance. What is inherited is not the actual hypersensitive state but a predisposition or tendency to get readily sensitised. On what exactly is this predisposition founded is obscure. Some hypothetical endocrine, biochemical, nervous and other peculiarities have all been called in support to explain the nature of the constitutional abnormality of the predisposed individual. In them the evidence of an unstable vasomotor mechanism may be apparent. Whether their predisposition is a general peculiarity of the entire organism or only certain sites and tissues are endowed with it, these serving as shock organs, is by no means clear. Whatever that be, on a back ground of genetic peculiarities the allergic state is readily implanted as a result of sensitising exposures to allergens and manifests itself by an excessive reaction when brought into contact with the specific allergen. The hereditary factor may develop in childhood or, what is more

common, may not appear for several years after birth. It may be unimportant, difficult to demonstrate or absent in certain types of allergy. For instance, certain types of bacterial allergy may not have any genetic relationship; at any rate, none is traceable.

Under human allergy are included a variety of conditions such as asthma, hay fever, allergic rhinitis, eczema, urticaria, contact dermatitis, serum and drug allergies, food and gastro-intestinal allergies and bacterial allergy. Excluding a few of these, like the last mentioned one, in which a hereditary taint is either absent or its presence difficult to establish, there is undoubtedly running through them all an undercurrent of genetic predisposition. In many of them, like asthma and hay fever, it may be easier to demonstrate the influence of hereditary factors; the term atopy is reserved by some to denote such conditions. No attempt is made here, and it does not seem very necessary for the present purpose, to draw any rigid distinction between atopy and other forms of idiosyncracy.

Methods of Sensitisation. Sensitisation occurs spontaneously in man. Opportunities for it may present themselves very frequently and in diverse ways; and man is constantly exposed to them. To trace the exact route, time or manner of sensitisation in every instance is a practical impossibility. But that does not invalidate the assumption that the various forms of hypersensitivity are essentially identical. The discrepancies can be explained satisfactorily. Moreover, the argument that in many cases any history of previous sensitising exposure is lacking has been weakened by the newer knowledge about the distribution of common antigens (p. 232). However, in a large number of cases the possible methods of sensitisation are apparent and when reactions occur their relationship to previous specific exposures can be sought out.

Spontaneous sensitisation may occur through inhalation, ingestion, skin contact or injection. In utero sensitisation of the offspring can take place with food substances, bacterial toxins and other allergens, transmitted from parent to progeny across the placenta. Passive sensitisation of foetus is possible and cases of it have been reported.

Contact with animals favouring inhalation of danders, inhalation of pollen, sleeping on feather pillow and working in factories where substances like ipecac, mercury, arsenic and others are

manufactured, all these afford ample opportunities for repeated exposures and consequent sensitisation to occur through the respiratory route. Many workers have shown that ingested proteins may be absorbed unaltered from the intestines. Obviously therefore, sensitisation with ingested food may be a common method; drugs ingested may also act in this way. The mystery surrounding the mechanism of sensitisation with drugs and other non-antigenic substances has been partly unravelled by the demonstration that such substances may unite within the body with proteins and form specific antigen complexes (p. 323). The repeated use of antiseptics, application of antiseptic remedies and drugs, insect bites, injection of serum or drugs are all frequent occurrences; so also infection with bacteria and other plant and animal parasites.

Notwithstanding such innumerable chances, only a small proportion of the exposed individuals develop sensitivity. Apparently, as alluded to above, their tissues are attuned to it as the result of some constitutional peculiarities. In them sensitisation may also be hastened and reactions undoubtedly precipitated by such non-specific influences like cold, excessive sweating, irritating fumes or dust and infectious diseases, like pertussis, pneumonia and influenza.

Passive Transmission. The passive transmission of human hypersusceptibility has been shown to be possible. This strongly supports the view that the essential mechanisms of experimental anaphylaxis and natural hypersensitiveness are identical. Transfer of the allergic condition to lower animals has not succeeded. This failure does not constitute any valid evidence to disprove the possibility of passive transfer from man to man. It can well be that the animals sought to be sensitised are insusceptible to those substances that were employed to create active sensitisation in man; these substances may not be fixed by the cells of the animals. On the other hand, there is some evidence to show that human allergy may be passively transmitted from man to man. Prausnitz-Küstner reaction is an example. The serum of a sensitised individual is injected intracutaneously into a normal person; 24–48 hours later, the specific incitant is injected into the same site as also into a normal adjacent area; in 10–15 minutes, a well marked urticarial reaction develops at the sensitised site, but no effect is produced on the control area, thereby

proving the presence of allergic antibody or reagin in the serum of sensitised subjects.

The sensitised individual spontaneously and specifically reacts on coming into contact with the allergic antigen. The allergens need not necessarily be proteins though many of them are. Their contact with the sensitised organ or tissues is brought about in manifold ways, the same as those subserving sensitisation. The clinical manifestations depend upon where in the body the sensitised tissues, shock organ, are located and also upon the nature of the offending allergen.

Different substances may give rise to the same symptom complex. For instance, egg white or shell fish gives rise to the same symptomatology. It is a well known clinical observation that the same allergen may give rise to more than one clinical manifestation. For example, pollen grains may cause either asthma or hay fever, affecting in the former the bronchial and in the latter the nasal mucosa. The symptomatology, therefore, differs in these conditions. Again, egg white may produce gastro-intestinal disturbances, urticaria or eczema, each with its peculiar site of affection and consequently differing symptomatology. One allergic manifestation in an individual may change into another. In the case of asthmatics, it is not uncommon to get a history of eczema at infancy or childhood. A migrain of early life may altogether disappear and give place to asthma at a more advanced age. When two conditions coexist in the same individual, for instance asthma and eczema, one of them remains entirely quiescent when the other is active. It should also be noted that the existence in a person of one type of allergy does not preclude the possibility of developing another type: the same individual may exhibit sensitiveness to more than one antigen at the same time. For example, asthmatics are easy victims to spasmodic rhinitis, horse serum allergy, food allergy, etc. or they may show sensitivity to drugs, like potassium iodide, or develop bacterial allergy.

The presence of hypersensitivity can be demonstrated by the intradermal injection of allergens or their extracts. A positive reaction is given by the appearance of a wheal and erythema. This furnishes a practical means of identifying the offending allergen, which enables steps to be taken for specific desensitisation.

The Allergic Antibody: Reagin. The exact nature of the allergic antibody (reagin, allergin) remains obscure. There is

no definite proof that it is different from other types of antibodies. The failure to demonstrate precipitin in the serum of allergic subjects has been cited as an argument in favour of regarding reagin as a separate substance; but it should be noted that hypersensitive reactions with a serum occur with very low concentration of antibodies, too low to form precipitate. Just as the chemical methods are relatively insensitive, compared to the precipitin reaction, for the detection of antibody proteins, so is the precipitation reaction compared to the hypersensitive reactions. Further, it is possible to induce passive sensitisation with sera in which no precipitin is demonstrable. These and other considerations suggest a close similarity between the allergic reagin and other antibodies. Between reagins and the anaphylactic antibodies some minor differences have been pointed out. An oft-quoted one is that the former do not passively sensitise guinea-pigs. But this fails to carry much weight, for even in the case of the anaphylactic antibody, sensitisation depends upon the type of animal. On the whole, no adequate justification seems to exist to consider reagins as substantially different from the anaphylactic antibodies.

Mechanism. The precise mechanism of the allergic reaction is not clearly understood. It is believed to be an antigen-antibody interaction with a cellular location, similar to that of anaphylaxis, and that the symptoms are due to a secondary histamine effect. There is some ground for this belief, at least in the case of certain types of allergy, but in others there is as yet no evidence to substantiate it. However, even in the latter, it is considered that the symptoms are due to the action of histamine liberated by the injured cells, though it is not evident how the trauma is caused.

Desensitisation. Desensitisation, albeit difficult, can be accomplished, but not to any point of complete effacement. By careful feeding or injection methods, most of the food allergies can be abolished or, at least, reduced in intensity. Likewise, encouraging results have been reported of having desensitised hay fever subjects by spraying their nasal mucosa with extracts of pollen grains and asthmatic subjects with extracts of food and other allergens.

The Gastro-Intestinal Type of Hypersensitiveness. Various kinds of food are responsible for inducing hypersusceptibility in gastro-intestinal idosyncracies. Milk, fish, eggs, meat and wheat

are the commoner ones, probably because they are the more common articles of diet. Sensitisation may occur in utero or after birth. In the latter case, it occurs only through the alimentary tract and symptoms are, therefore, largely referable to it, thus exhibiting a tendency for the localisation of reaction. Whether there is a generalised sensitisation, but giving rise to symptoms chiefly related to the alimentary tract because of the primary intimate contact of the allergen with the gastro-intestinal mucosa, or whether the alimentary mucosa is sensitised in a greater degree than other parts of the body, remains an open question.

Immediately the offending or allergic food is taken by the supersensitive individual, swelling of the lips and tongue and difficulty of swallowing ensue in some cases. It may stop with these or, in severe cases, abdominal discomfort, nausea and vomiting may supervene. Colic and diarrhoea may set in after a few hours. General symptoms, such as rapid pulse and low blood pressure, are not uncommon. Skin conditions, like urticaria, are common features of food allergy; puffiness of the face is common, but angioneurotic oedema is rare; eczema is a chronic manifestation. Asthmatic attacks or coryza may be set up.

The Respiratory Type. The chief agents responsible for this form of allergy are pollen, house dust and emanations from animals and plants. Dandruff and hair of horses, dogs, cats and other animals, feathers, house dust, flour of cereals, and industrial dust are the common ones. Hay fever, asthma and spasmodic rhinitis are the important ailments caused by these. When pollen is the incitant, a seasonal variation is naturally exhibited in the incidence of the concerned disorders. Non-specific factors, like cold, excessive heat and sweating and sudden entry into stuffy rooms, favour the incidence of reactions more in this than in any other form of allergy. So also the administration of horse serum and the ingestion of food allergens will unfavourably influence, starting reactions or aggravating them.

Drug Hypersensitiveness. Intolerance to certain drugs is a common abnormality. There may be history of habitual use or constant exposure, but often reactions follow primary contact and it is difficult or impossible to discover any sensitising exposure in such cases. Large number of drugs are found capable of exciting allergic reactions; quinine, aspirin, arsenobenzol compounds, potassium iodide, salicylates, mercury compounds and

lysol are some of them. They are introduced into the body or come in contact with it through any of the routes, ingestion, inhalation, injection or cutaneous contact. It may be that the exciting property resides either in the whole molecule constituting the allergen or in certain groups of the molecule. For example, individuals intolerant of potassium iodide may, nevertheless, tolerate sodium iodide.

Symptoms of drug idiosyncracies are not mere exaggerated pharmacological reactions of the concerned drugs but are true allergic manifestations. Skin is the most frequently involved site and cutaneous eruptions, such as urticaria, eczema and dermatitis, are the commonest expressions of drug allergy. But gastro-intestinal and respiratory tracts may also be the seats of sensitisation when symptoms, differing little from those due to other types of supersensitiveness, are produced.

These drug allergens are immunologically simple substances having no claim to any independent antigenic property. This is no more a hindrance to the inclusion of drug intolerence in hypersensitiveness. The study of synthetic antigens has considerably elucidated this problem. It has shown that antigenically inert substances, when combined with proteins, give birth to complex compounds, behaving like foreign substances and endowed with high antigenic potency, capable of provoking antibodies and causing sensitisation (p. 233). Such chemical substances impart specificity to the complex antigen and also function like bacterial haptens, combining specifically with the antibodies incited by the conjugated products. This may be happening also in drug allergy. In predisposed persons, these simple compounds or their active cleavage products may after repeated contact unite with the body proteins and form complete antigens; because of the union these proteins have lost their identity and become immunologically foreign to the body. Such drug-protein complexes may sensitise the tissues which would then respond specifically on a subsequent contact with the drug. Drugs may thus play the role of haptens. This may explain the allergic propensity of certain drugs which intrinsically lack immunising or sensitising properties.

There is a miscellaneous group of substances which cause hypersensitive reactions and in this group are included certain chemicals like mercury and lead, insect powders, cosmetics, plant irritants, like ivy and nettle, caterpillar irritants, various insect

bites and others. Usually they cause skin erruptions, like urticaria and dermatitis, and rarely manifestations referable to other systems.

Serum Sickness. Toxic reactions may follow serum therapy not only in persons previously injected with horse serum but also in a certain percentage of individuals after primary injection. In the latter there is the possibility of previous sensitisation by the intestinal route and so both types of reactions may possibly constitute a single allergic group. The possibility of previous sensitisation during immunisation against diphtheria with toxin-antitoxin mixture should not be lost sight of. Serum reactions are specific; they are due to horse serum, *i.e.* to the proteins present in it, and not due to the contained antibodies.

An acute and fatal type of reaction having an anaphylactic pattern is extremely rare. What is common is the relatively benign serum sickness. In this a characteristic train of symptoms appear 6–10 days after the administration of immune horse serum. They are skin erruptions, usually of the urticarial or scarlatiniform type, oedema of face or other parts, joint pains, general disturbances like slight rise of temperature and malaise, mild albuminuria or, occasionally, oliguria, enlargement of glands and an initial leucocytosis followed by slight leucopenia with relative lymphocytosis. These may go on for one or two days to a week or more.

Without any reinjection symptoms may recur after an interval of a week or ten days. Most likely, it is due to the presence of multiple antigens in the horse serum each with a different incubation period.

The incubation period shortens after subsequent injections and symptoms tend to be more severe. Such reactions have been named 'accelerated reactions'. When repeated injections are given at shorter intervals, reactions follow in a few hours and these are referred to as 'immediate reactions'. Both these types of reactions serve to show that man can be readily sensitised with horse serum. If injection is given within a short time after the incubation period, a local reaction may develop analogous to the Arthus phenomenon. Serum reactions are particularly serious in asthamatics, especially in those who develop the disease on coming into contact with horses, the so-called horse asthmatics.

Serum reactions are seldom fatal, but, as mentioned above, they may be serious, especially in those with asthmatic tendency. They may be prevented by desensitisation, although the effects of it are neither complete nor lasting. Hence, enquiries into the presence of sensitivity and attempts to desensitise the positive case should be a first essential in serum therapy. But serum should not be withheld in urgent cases on the plea of the possible development of serum sickness; for the danger from this is much less serious than that from the original disease. Serum reactions should be carefully guarded against and particular care should be taken in the treatment of horse asthmatics with horse serum. Any history of such diseases as asthma, urticaria or eczema or of previous serum injections should be diligently sought for.

The presence of serum hypersensitivity can also be elicited by certain tests. In the ophthalmic test, one drop of undiluted horse serum is instilled into the conjuctival sac. Sensitivity is indicated by itching, lacrymation and reddening, all appearing within thirty minutes. In the skin test, 0·1 c.c. of a 1 : 100 dilution of serum is injected subcutaneously with another injection of normal saline solution as control. In positive cases an urticarial wheal develops at the site in 5–20 minutes, subsiding in an hour or two.

The process of desensitisation consists of the repeated administering of very small doses of serum at short intervals. The patient is given an initial dose of 0·5 c.c. of a 1 : 100 dilution (with sterile isotonic salt solution) of the serum subcutaneously. If no reactions ensue, double the dose at the end of 15 minutes. Repeat this procedure, doubling the previous dose every time until a dose of 1 c.c. of neat serum can be administered producing no reaction. There is no standard technique and suitable alterations in the quantity and timing should be made according to the individual's reaction. Half an hour after the last dose of the series, the full dose may be given in one injection by any of the routes except the intravenous. Should it be necessary to give serum by this route, a further desensitising process should be gone through. For this 1 c.c. of a 1 : 10 dilution is given cautiously and slowly; half an hour later, twice the amount and so on until the full dose is reached. Adrenaline should always be handy to meet any emergency. When desensitisation has to be waived due to urgency and serum has to be given intravenously for the

same reason, an alternative method is the administration of 0·25–0·5 c.c. of adrenaline along with the serum; adrenaline does not interfere with the therapeutic value of serum.

Serum should not be given intravenously to patients with positive skin test without desensitisation. Intravenous administration should as far as possible be avoided in asthmatics and in those with a positive ophthalmic test, unless urgency demands as in malignant diphtheria.

Remedies usually employed against serum sickness are ephedrine, adrenaline and calcium. But definite evidence is wanting that they have any preventive value in serum sickness. Histaminase, a ferment obtained from the intestinal mucosa, has been reported upon favourably both in the prevention and control of serum sickness. It may be given orally or by injection, the latter being more efficacious. But the most effective way of obviating serum sickness is by the use of refined and concentrated immune serum or by using serum prepared in another animal.

Bacterial Allergy. Bacterial allergy is the term applied to human hypersensitiveness caused by bacteria or their products. It is not characteristic of all types of infections. We have seen that microbial infection stimulates the production of protective antibodies, antitoxic, antibacterial or both, which in some way overcome the noxious agents. Yet another feature of infection is that bacterial proteins may act as sensitising agents. They induce hypersensitiveness with no apparent relation to immunity and sometimes in addition to it. The condition develops in the course of infection and expresses itself in the form of cutaneous reactions, of the tuberculin type, on injection with the specific allergen. Infected animals also respond in a similar manner. These skin reactions are utilised as means of diagnosis. Allergy of infection also seems to play an aetiological role in certain diseases like asthma and rheumatic fever.

Bacterial allergens are protein in nature. They may also cause anaphylaxis (bacterial anaphylaxis), similar to protein anaphylaxis. Bacterial haptens are unable to sensitise but are capable of inducing allergic reactions specifically in the sensitised animal or man, recalling their behaviour in immunity reactions.

The precise mechanism of bacterial allergy is by no means clear. Nor has it been proved that an allergic state develops in all types of bacterial infection. It is easy to demonstrate the role

of allergy in some infections like tuberculosis, but the same cannot be said of others. Extensive experimental studies have been made about the *tuberculin reaction*, but still its intimate mechanism of production or its bearing on immunity in tuberculosis is not clearly understood (Chapter XXVIII). However, these experiments tend to show that the tuberculin reaction is not merely an infection phenomenon but depends largely upon the occurrence of inflammatory cellular reaction and formation of tubercles. As has been suggested, such modification of the allergic state by the inflammatory reactions incidental to them may be a common factor in all bacterial allergies.

As indicated above, the best known example of bacterial allergy is found in tuberculosis and the tuberculin reaction is taken as the representative of the group. It is delayed in its appearance, persistent in duration and considerably modified by inflammatory cellular reaction. Thus, it displays certain differences from the reactions produced by non-bacterial agent. The latter are usually characterised by an immediate wheal formation associated with erythema, both disappearing rapidly; they are hardly complicated or influenced by inflammatory reactions. Nevertheless, both types seem to be substantially same and the differences are explainable by the complexity of bacterial antigens.

Other reactions occasionally employed in diagnosis are the *mallein reaction* in glanders, *brucellin reaction* in brucellosis and *Frei test* in lymphogranuloma inguinale. *Trychophytin test* in trychophytosis is an example of allergic test in a fungus disease, and *Casoni test* in hydatid infection is an example of allergy created by a metazoan animal parasite.

These allergic tests are not to be confused with the group of skin tests represented by the Schiek reaction; the latter type is based on immunity response—the neutralisation of injected toxin by circulating antitoxin.

It is believed, mostly on clinical grounds, that infection of the respiratory tract and also of the nasal and paranasal sinuses plays an important aetiological role in asthma and allergic rhinitis. Bacterial products from these foci are regarded as causing sensitisation as the result of long continued action. How exactly bacterial sensitisation plays its part in the causation of these disorders, is still obscure. Similarly, in rheumatic fever streptococcal sensitisation has been postulated as the underlying factor.

In this the susceptible tissue is said to be the fibrous tissue frame work, particularly of the heart, the subcutaneous tissue and the synovial membranes, an acute inflammatory condition of which forming the basic pathology. Focal sepsis, including throat infection, is also considered to be important in the causation of the condition by furnishing streptococcal products for sensitisation. The view has been recently advanced that the acute manifestations are the outcome of superinfection with the haemolytic streptococcus which probably supplies the toxic dose. There is in rheumatic conditions, as also in experimental rabbits, definite evidence of enhanced cutaneous sensitiveness to streptococcus: the presence of streptococcal antibodies in the patient's blood has been likewise demonstrated (p. 382). But these proofs, imposing as they are, are insufficient to establish the allergic hypothesis and the matter still remains controversial. Similarly, scarlet fever has also been suggested to be due to streptococcal sensitisation. But the evidence on its behalf is still less satisfactory.

CHAPTER XIV

BACTERIAL VARIATION

Variation is a characteristic of all forms of life. Bacteria are no exception to it. Variation among these lowest forms of life has been known almost from the beginnings of bacteriology. In fact, one of the earliest conceptions regarding bacteria was that they underwent during growth spontaneous and extreme changes in form and function. Interchange between coccal, bacillary and spiral forms was looked upon as the normality among micro-organisms. In this early idea lay the genesis of the theory of *pleomorphism* later postulated by Nägeli in 1877, who maintained that all bacteria were members of possibly but one single species and that the different forms encountered were all variants. The pleomorphic concept did not restrict itself to morphological changes alone but necessarily included in its scope alterations in virulence, proteolysis and other functions as well.

The doctrine of pleomorphism held sway until the development of pure culture techniques of bacteria by Koch and others. Following the new technique a large number of bacterial types, greatly differing in form and function, were isolated in pure culture. On closer study, these individual species were found to exhibit a remarkable constancy in their morphological and functional characters. Thus, Koch and his collaborators were able to demonstrate clearly that the extreme variability of bacteria observed in fluid cultures was but apparent and not real, arising from a fallacious interpretation of impure cultures. With the acquisition of this newer knowledge regarding bacterial species, the pendulum was swung violently to the other extreme and there arose the *monomorphic* theory, as rigidly held by Cohn, Koch and others as the pleomorphic conception was by its protagonists. According to this doctrine, each bacterial species had its fixed and unalterable normal form and the so-called variant forms encountered in old cultures or in tissues were either contaminants or abnormal products relegated as *involution* or *degenerative* forms

occurring preparatory to death. With the further development of knowledge these extreme views gave place to the modern conception of bacterial species as possessing a very large amount of stability in their specific attributes which breed true to type but are nevertheless subject to variations of a transient or persistent character. There is, however, a school of contemporary thought which, while recognising considerable stability in the specific characteristics of micro-organisms, as taught by the monomorphic school, postulates the existence of a complex life cycle, cyclogeny, for bacteria. Many of the so-called variations, according to them, are but cyclogenic changes. But this view does not obtain any wide acceptance among bacteriologists in general (*vide infra*).

Variation as a problem, therefore, did not exist in the purview of the early bacteriologists. Alterations in characters, to them, were either normal phenomena or teratological processes deserving no special consideration; nor in any case did they appear as having any significance in the relationship of bacteria to disease. No attention was, therefore, bestowed on the study of variation from a biological aspect. But more recently, with the recognition of the fallacious nature of the above doctrines, fresh interest has been roused in the subject of bacterial variation, and during the last two or three decades extensive researches have been made to unravel its cause and significance to the bacterial unit. These efforts have not yielded much success and today we are almost as ignorant as ever regarding these aspects of variation, although we are in possession of a wealth of knowledge about the various manifestations of variability. With every advance of knowledge, fresh problems of variability present themselves, indicating that the field of variation is vast and varied. Numerous instances have been disclosed of variation of a temporary or semi-permanent nature not only in the morphological and biochemical characters but also in virulence, toxigenicity, antigenic nature, pathogenicity and immunological response, so that the subject of variation has assumed a very complex nature. It is but logical to conclude that some of these variations must have by nature important bearing on the problems of medical bacteriology. How far such variant changes, observable in the culture tube, are correlated with the incidence and evolution of disease processes in the individual as well as on the community is still unsolved, demanding yet a vast amount of investigation. The significance of variation in

relation to the procedures of immunisation and serological diagnosis will become amply evident as we proceed.

Variation means deviation from a common type, whether temporary or lasting. Normally, the species breed true to type. But at certain times and under certain conditions bacterial cultures are seen to manifest changes in one or more qualities from the original type. It is axiomatic that we should have precise knowledge of the modal characters pertaining to the common type before we may proceed profitably with the study of any alterations from them. Such knowledge regarding bacterial units is largely wanting due to the inherent difficulty of studying these fast-multiplying microscopic forms of life. It may be reminded that immunerable generations occur in the course of twenty-four hours (10^7–10^9 viable cells) and to follow an organism through all these is an all but impossible task. Also, there is a sharp cleavage of opinion among bacteriologists regarding certain vital aspects of bacterial physiology. Some workers believe that bacteria possess a complete life cycle with all the phenomena of a sexual mode of reproduction, while the majority of bacteriologists reject such a contention (p. 342). Unanimity of opinion is also lacking as to what should form the basic criteria of species. The result of all these is that different workers approach the subject with different biological standards. Naturally, interpretations are bound to vary. It may also be noted that when we talk of variability we invariably take the ordinary culture products as the natural forms. This may not be altogether justifiable. At least in a few cases, for instance the nitrogen-fixing bacteria, the morphology of bacteria as occurring in nature displays substantial differences from their morphology developed in artificial cultures.

Variations may occur in morphology, physiology or in both. Morphological variations include modifications in shape, size, staining properties, cell grouping and in the formation of flagella, capsule and spore. Variations in these respects are of frequent occurrence; they are often temporary in character. Similarly, variations in size, shape and appearances of colonies may occur. What constitutes the natural impelling force behind variability, is difficult to tell; some changes may be a direct response to certain environmental stimuli. Such changes are not true variations but only temporary adaptations incidental to the normal life of bacteria as of any other living thing.

Spontaneous alterations in the size of the individuals of a species are often observed. For example, longer and shorter forms of the same bacillary species may frequently occur in culture. Such variations are usually temporary in character, but it has been found possible, in some cases at least, to convert a temporary change into a hereditary one, that is, a change in which the variant quality is transmitted to succeeding generations even in the absence of special conditions that originally produce the change. By selective subculture from a culture of *Bact. coli* of individual cells that grow to unusual lengths, it is possible to raise a culture of purely long forms which breed true to type for many successive generations, showing no tendency to return to the original short form. A character variation is thus impressed upon the organism.

Numerous other instances can be cited of departures from the normal morphology of bacterial species, which may occur naturally at some time or other during their life. Bacillary forms may even assume coccoid forms, on the one hand, or mycelial forms, on the other. The appearance of elongated or diphtheroid forms of streptococci and pneumococci in their cultures is an oft-noted phenomenon. Variations may also be encountered in cell grouping. As described in a previous chapter, the arrangement of cells in some particular fashion is a characteristic of several species of bacteria. Spontaneous deviations from the usual pattern are often noted; for example, streptococci may grow in pairs and *D. pneumoniae* in chains. Again, a flagellated variety may for some reason become suddenly non-flagellated. This is the predominant feature of the H–O variation, in which a spontaneous suppression of the power of forming flagella results in the production of non-motile variants (p. 347). Another example of morphological variation is the suppression of capsule formation in the capsulated species, such as the pneumococcus.

A spore-bearing microbe may likewise cease to develop spores at certain times or under certain conditions. The anthrax bacillus, for instance, may become spontaneously asporogenous. Such naturally occurring sporeless variants seldom show any tendency to reversion. Pasteur found that cultivation of the anthrax bacillus for several days at a temperature of 42·5°C. resulted in the loss of virulence and the appearance of asporogenous variants. The same can be accomplished by growing

the organism in the presence of dilute antiseptics. While the heat-induced sporeless variants show great reduction or loss of virulence, the same correlation between sporogeny and virulence is not always seen. In other words, loss of virulence is not a constant feature of loss of ability to form spores. Virulence does not rest merely on spore-formation. Virulent strains may be sporogenous or asporogenous; so also avirulent strains.

Of late, the nature of bacterial colonies formed on solid media has assumed special importance. Their size, shape, margin, general configuration, surface features, consistency and colour may all display at some time or other deviations from the generally accepted parent type. The character of growth in fluid media may likewise show modifications spontaneously or under certain conditions. All these are dealt with fully in later sections.

Filterable Forms of Bacteria. Bacteria are, as a rule, non-filterable. Sometimes filter-passing forms, capable of propagating themselves and under certain conditions developing into the parent form, are said to develop naturally in the culture of certain bacterial species. Some workers assume that they represent a filterable phase in the life cycle of bacteria. It has even been suggested that the causative agents of some of the virus diseases represent a filterable stage in the life cycle of certain bacteria; for instance, the poliomyelitis virus has been claimed to be a filterable phase of the streptococcus. Many workers, however, doubt the existence of filtrable forms and attribute their presence to faulty technique in filtration. The data available at present regarding their occurrence are far too meagre to carry conviction.

Of some interest is the observation made on the so-called G (gonidial) forms of bacteria. They are granular elements formed by bacteria and believed to be capable of independent multiplication into the same form and eventual transformation into the original type. Many observers have noted their presence within bacterial cells. When liberated from the cell, they are readily filterable through bacterial filters and from the filtrates microscopic colonies, G colonies, never more than 0·2 mm. in diameter, grow. Such colonies are composed of small elements of diverse morphology with Gram-positive staining character whatever be the staining property of the parent type. In their biochemical properties the G forms are quite inert and different

from the original forms; they are also reduced in pathogenicity. As indicated above, they finally revert to the original type, but the process is slow. Some workers maintain that they constitute organs of asexual reproduction, forming a regular phase in the life cycle of bacteria. However, many bacteriologists do not agree with this view and, pending more exhaustive studies, no definite conclusion can be arrived at regarding their occurrence or significance.

Physiological Variation. Variations affecting almost every physiological character of bacteria may occur spontaneously. They may be temporary or more lasting in character. Physiological modifications may also be induced by experimental procedures. Many of the functional activities of bacteria, it may be recalled, are based on enzymic mechanisms and so physiological variation largely implies variation in the enzyme production. Changes in the fermentative, proteolytic, haemolytic, chromogenic and other properties have all been observed. Variation usually takes the form of an acquisition or loss of one or other of these properties by the variant. A variant, for example, may develop the capacity to ferment sugars not previously attacked or lose a particular fermentative faculty which the parent form possessed. Such gain or loss of a quality may or may not be productive of associated changes; often it is. Whether the disappearance of a character is due to a complete loss and its reappearance due to a gain *de novo*, are questions which are inherently difficult to prove. When there is apparent loss, the quality in question may be assuming latency without complete extinction such that it can re-establish itself when conditions become suitable.

Another variation in the physiology of bacteria is the alteration in the pigmentary metabolism, which the chromogenic bacteria may at times exhibit. The power of pigment production of a particular species may sometimes partially or completely disappear, the growth appearing poorly coloured or colourless altogether, and reappear after varying intervals with restoration of the original, or normal, hue to the colonies. For example, *albus* variants are occasionally given birth to by *Staphylococcus aureus*; growth with white, pink or red colour may be produced by selective subculturing of *Chromobacterium prodigiosum*. Such changes are often environmental in origin, but in some cases no cause can be traced.

Adaptive and Constitutive Enzymes. Bacterial enzymes can be placed in one of two classes: enzymes that depend upon the presence of suitable substrates for their production and enzymes that are formed independent of the presence or absence of such substrates. The former are called *adaptive* and the latter *constitutive* enzymes. The production of adaptive enzymes is thus a variable factor, although the capacity to do so is as much a permanent quality as the capacity to produce constitutive enzymes. The stimulating influence of a specific substrate is necessary for the elaboration of adaptive enzymes in appreciable quantities. In other words, the ability of an organism to utilise certain substrates undergoes marked accentuation when it is cultivated in the presence of such substrates. In the absence of these substrates, probably the organism is simply inactive in this respect; it cannot be said that the specific capacity is entirely lost; only any detectable amount of enzymes may not be produced; there is no conclusive evidence to say that the enzymes are not produced at all. The tryptophane-utilising enzyme of *Bact. coli* is an example of an adaptive enzyme. Washed suspensions of this germ grown on a medium containing tryptophane will attack this amino acid much more readily than washed suspension from a medium not containing tryptophane. As mentioned elsewhere, many of the proteases belong to the adaptive type. In the absence of proteins, these proteolytic enzymes are formed only in minimal quantities. But when provided with suitable proteins, the enzymic activity is very much enhanced. The production of constitutive enzymes, as noted above, is not conditioned on the substrate environment. The enzyme of *Bact. coli* concerned in the decomposition of glucose illustrates this type of enzyme; the colon bacillus will readily attack glucose whether or not it is previously acclimatised on media containing this sugar.

The enzymic variation described above may be rapid or slow in appearance. In some cases a single transplantation into a suitable medium will restore marked enzymic activity, while in others several transfers on a medium containing the particular substrate are necessary to bring about the change to an appreciable extent. In any case an essential prerequisite for adaptation is the utilisation of the substrate by the organism and cell multiplication; without these the process of adaptation does not materialise in the vast majority of cases. From the above it is

evident that these enzymic variations are determined by the bacterial environment; they are, therefore, to be regarded as temporary adaptations to external stimuli.

A more persistent type of variability in the biochemistry of bacteria is that observed in *Bact. coli mutabile*. This is a non-lactose-fermenting strain of colon bacillus. When cultivated on a lactose-containing medium, like MacConkey's agar, early colonies are all entirely white. On further incubation red papillae appear on the white colonies, indicating that some of the bacilli have suddenly developed the capacity to ferment lactose with the production of acid. Organisms from the red papillae breed true to type, growing into entirely red colonies which display no tendency to return to the original non-lactose-fermenting type. Subcultures from the white portions of the original colonies reproduce white colonies which, as before, give birth to red papillae. Laborious studies recently conducted have brought to light that one in a hundred thousand cells of this organism grows into a lactose-fermenting variant. The nature of this change is not understood; in its suddenness and permanency it resembles mutation.

Several experimental variations have been similarly produced in members of the colon-typhoid-dysentery group of organisms, the resulting variants in all such cases exhibiting varying degrees of permanency. Thus, a dulcite-fermenting strain of *Bact. typhosum* has been reared up, although it does not display the same tendency to non-reversibility as the lactose-fermenting variant of *Bact. coli mutabile*. Probably analogous to these adaptations are the nutritional changes that may be induced in certain pathogenic bacteria. It has been pointed out in a foregoing chapter that the typhoid bacillus, though requiring preformed tryptophane for its growth, can, nevertheless, be trained to synthesise this amino acid from ammonia. Likewise, by training, the dysentery bacilli can be made to grow in the absence of nicotinamide which is normally essential for its growth. Several such changes are on record in which nutritionally fastidious organisms have been rendered less exacting or non-exacting by training and adaptation. It is a common observation that many pathogens on first isolation are poor growers on the common laboratory media, but sooner or later they become accustomed to them as the result of subculture. So also, the gas requirements

of certain species can be varied by repeated subculturing. *Br. abortus*, for example, requires a high CO_2 tension for primary isolation, but after a number of subcultures it learns to grow under ordinary CO_2 tension.

Antigenic Variation. Variations in the antigenic make-up of bacteria are by no means uncommon. The subject has assumed considerable importance of late and it will be considered more fully under bacterial dissociation (p. 343). Antigenic variation may not be an isolated phenomenon; it often appears in association with morphological and cultural variations and often apparently as a sequence to these. For example, when a flagellated species loses its ability to produce flagella, as in the H–O variation, it also inescapably loses therewith the flagellar antigen. Likewise, when a capsulated organism loses the power of developing capsule it inevitably suffers a loss of the capsular antigen. The loss of flagella or capsule leads to important cultural changes. The antigenic individuality of an organism is inseparably bound up with its chemical structure. Hence, alterations in these two go hand in hand. On the antigenic structure generally depend several other properties such as virulence, toxin production and serological response. Hence, any change in the antigenic constitution is bound to have its reflection in such dependent properties. For instance, when a capsulated strain of pneumococcus has undergone variation in the form of loss of the capsular antigen, its chemical integrity has suffered, serological specificity has disappeared and its virulence and pathogenicity have undergone profound degradation changes. The new non-capsulated variant, left mainly with the species-specific nucleoprotein, is antigenically different from the capsulated original form. It is also different in its cultural characters. Thus, antigenic variation is often associated with a train of changes, the nature of whose underlying mechanism or mechanisms is unknown. Variation in the antigenic structure of micro-organisms may also occur unaccompanied by any other changes, for instance the phasic variation of the *Salmonellas*. Antigenic variation may be temporary, as for instance the phasic variation of the *Salmonellas*, or more enduring like that involved in the S–R variation (p. 343).

Variation in Virulence and Toxigenicity. The loss or lowering of virulence, or *attenuation*, is a well known phenomenon (p. 202). The opposite process is called *exaltation* of virulence. The

pathogenic microbes in their natural host tissues usually exhibit a high degree of virulence. But through long sojourn in the host they have been observed to lose their virulence partly: strains isolated from carriers may be less virulent than those that cause infection. Reports that attenuation may be forced upon in vivo by the defence mechanism of the body should, however, be critically assessed. It is a question of supreme practical importance, especially in the field of epidemiology. Outside, in the culture tube a considerable fall in virulence is usually experienced after repeated subculture. Thus, artificial cultivation itself, not involving the introduction of any unfavourable growth conditions, reduces virulence in course of time. In fact, the readiness with which virulence is lost in artificial cultures is a great handicap in experimental work. By aging, the culture of a virulent organism becomes weakly virulent or avirulent; probably the metabolic products of the organism itself may have a hand in this transformation. Further, the pathogens may also be forced to lose their virulence by cultivating them under adverse conditions; impoverished nutritional surroundings, unfavourable temperature conditions and inclusion of antisera in the culture media may all bring about a lowering or loss of virulence. Examples are the B.C.G. strain of *Mycobacterium tuberculosis*, which is a permanently non-virulent variant, and Pasteur's attenuated anthrax strains. Attenuation can also be brought about by serial passage through unfavourable host species; an outstanding example is the conversion of smallpox virus into vaccinia virus by passage through cows. Another attenuating agent is the bacteriophage.

It is hard to induce exaltation of virulence in artificial culture, but passage through suitable animal species offers better success in many instances. Thus, serial passage through mice enhances the virulence of the pneumococcus or of the pestis bacillus. The enhancement of virulence by this process has been ascribed to a selective survival of the more virulent individuals in the body and the destruction of the less virulent or non-virulent ones by the action of tissue fluids and cells. This, of course, presupposes a disparity of virulence among the individuals in a culture; a few unattenuated organisms may be remaining intact in the degraded culture.

The ability to produce toxin may also suffer loss or diminution under certain conditions. Where toxin production and

virulence go together, as in *C. diphtheriae*, degradation in virulence manifests itself as a loss of the toxigenic power. But, where these properties are independent of one another, as in streptococci, any interference with the one need not necessarily affect the other. Variation in the toxigenicity of different strains of the same species is by no means unusual; the occurrence of different strains of *C. diphtheriae* yielding varying amounts of toxin is too well known.

Attenuation is often, but not always, associated with morphological and other changes. Loss of virulence of pneumococci, for instance, goes with the loss of ability to form capsule. This association, is not always constant. Variation in virulence may be seen with no other recognisable changes. Non-virulent non-toxigenic strains of the diphtheria bacillus, morphologically and culturally indistinguishable from the virulent toxigenic strains, are occasionally seen. Similarly, avirulent spore-forming anthrax bacillus may be encountered as well as virulent spore-forming strains. Variations in virulence and toxigenicity are also associated with changes in the behaviour of the organism to the action of phagocytes and bacteriophage. Loss of these properties is also accompanied by a loss of resistance to phagocytosis (p. 300); so also by a loss of sensitivity to the lytic action of bacteriophage.

Immunological Variation. It is a common observation that different strains of the same bacterial species display marked differences in their antigenic efficiency, that is, the capacity to provoke rapidly and effectively immunological response in the host. For example, certain strains of typhoid bacilli are efficient immunisers, while others are not. Per contra, some strains are highly agglutinable, while others are poorly so.

Variation in the immunological nature of bacteria is an important manifestation of bacterial dissociation. As already pointed out, it is intimately bound up with the antigenic variation. Within the same bacterial species there may be different immunological types, depending upon the presence of different antigenic moieties. For example, pneumococci are divisible into many immunologically unrelated types, the immunological peculiarity or specificity residing in the substance of the capsule. In the complex S–R change the loss of capsule is a prominent feature, obviously then, with this loss, that is of the specific capsular

substance, will also vanish the type specificity linked with the capsule, the rough variant being devoid of any type; it is now common to the whole species.

The Nature and Significance of Variation. Earlier in this chapter we have seen how the early pleomorphic conception of bacteria taught that bacterial species did not have any fixed basic attributes but were subject to unlimited changes. According to this conception the so-called variants had no special significance; they were but normal phases. Nor had bacterial variation, as we know it today, any place in the later monomorphic theory of bacterial species. In fact, this conception with its teaching of the immutability of bacterial species precluded the possibility of bacterial variation occurring. To the followers of this theory there were only normal and abnormal forms of bacteria; in other words, all unusual forms were regarded as pathological in origin. Thus, whether it is the pleomorphic or the monomorphic doctrine that held the field, variation as a phenomenon ***suis generis*** did not exist in the minds of the early bacteriologists.

Fluctuating Variation. We are still ignorant of the precise nature or mechanism of variation. Several views have been advanced. Variations were regarded by some as merely fortuitous in character, occurring at random with no obvious cause or significance. Many of them appear to be of a fluctuating type; they readily swing to and fro from a modal type. Although there is no permanency about it, it is, nevertheless, possible by selective methods to impart a degree of stability to a variant character, for example the creation of a giant cell culture of *Bact. coli.* (p. 332).

Mutation. Another view is that bacterial variation is of the nature of mutation occurring in higher forms of life possessing a sexual reproductive mechanism. Mutation implies genetic changes; it is hereditary and permanent in character. In the conception of mutation have also been included by some bacteriologists abruptness of change and irreversibility or permanence. Certain bacterial variants, *e.g.* the lactose-fermenting variant of *Bact. coli mutabile*, display both these characters. On evidence such as this some investigators believe that bacteria possess genes controlling all heritable characters and that genetic changes underlie all bacterial variations. But the premises are hypothetical; the question of the presence of nucleus and chromosomes is still

controversial and the evidence in support of a sexual mode of reproduction among bacteria is extremely slender. Hence, to invoke gene-mutation to explain the underlying mechanism of all bacterial variations is unwarranted in the present state of knowledge. But it cannot be denied that there are certain types of bacterial variation which closely resemble mutation and so the possibility of mutation as the basic mechanism cannot be altogether ruled out. The question can be settled one way or other only in the light of further data regarding the intimate structure of bacteria.

Adaptation and Impressed Variation. A widely held view is that variation is environmental and selective in origin. Environment undoubtedly plays an important part in the genesis of variants. There is ample evidence to show that alterations in the morphological or physiological properties of bacteria may all be induced by unusal conditions of life. Environmental variations may be temporary or more lasting. Temporary modifications are the result of the direct influence exerted by the environment; that is, they are temporary adjustments to suit the new culture conditions which are unfavourable for normal growth, and ready reversion to the original form follows the withdrawal of such unfavourable conditions; no more are the new characters handed down from parent to progeny. Some investigators believe that even in such circumstances hereditary transmission of characters may occur, at least, in rare cases. But it is doubtful that a direct action of environmental influences can alone give birth to more than fugitive modifications. Environmental factors that may necessitate adaptive changes are a higher than optimum temperature, high salt concentration, reduced surface tension, nutritional deficiency and the like.

Where variation is more lasting, a selective survival of the variants and a transfer of the variant qualities to the future generations seem to be involved. The changes are not of the nature of temporary adaptation, and coming back of the variant to the original type is not in the pattern of impressed variation. Instead, the process is one of selection of the qualities advantageous to the organism and a progressive modification towards a better type. The influence at work here is the favourableness of the environment in promoting the growth of special types of preformed variants in a selective fashion. In some way or other

variants are formed in the culture medium and those variants, which are presumably best fitted to the new surroundings, multiply rapidly overgrowing others which are suppressed and may finally become extinct. That is a selection of the fittest. What impels the appearance of the initial variants is by no means clear. Whatever that be, such selective transformation is progressive and tends towards permanency. We have no knowledge of the nature of the bacterial mechanism through which the environmental factors operate in the perpetuation of the variant qualities.

Bacterial Life Cycle. It has been postulated that bacteria may possess a complex life cycle, exhibiting a succession of morphological types, such as the normal forms, the involution forms, gonidial forms and gametes with sexual processes, and eventually restarting the whole chain of events in an orderly way. The evidence in support of a cyclogeny is very meagre. It may be conceded that certain rare morphological phenomena, like the spore-vegetative cell succession, have some semblance to the stages of a life cycle. Excluding such exceptions, no change has been so far shown to occur in any bacterial unit, which is indisputably cyclogenic in character. The upholders of the above theory claim that some, at least, of the variations are cyclogenic. Such variations, they say, occur regularly in the natural course of a complex life cycle of the bacterial unit and if we do not observe them regularly it is either because our opportunity for observation is restricted, or the cells in some of the phases are too minute to be resolved by the microscope or we are generally observing only one of the morphological stages of the organism, as others are suppressed in the constant cultural environments of the laboratory. Suffice it to say that this claim rests on a basis which itself has yet to be established.

The significance of morphological variations to the bacterial cells is obscure. The survival value of bacterial attributes is not clearly understood, so that the correct assessment of any particular gain or loss has perforce to rest on uncertain grounds. In what way a bacterium is affected by a loss of flagella or capsule or how the acquisition of some fresh physiological character benefits in the economy of bacterial life, are questions for which we are at the moment unable to give any definite and satisfactory answer. In view of the wide adaptive powers of bacteria many of the physiological variations may not be of any great consequence.

If any particular enzymic system, for instance, is lost some other mode of meeting the loss is invariably developed and the organism does not apparently seem to suffer any biological set back. Such is the elasticity of the metabolic apparatus of bacteria. Besides, most of the physiological variations are probably of the fluctuating type; they appear and disappear, never remaining permanent. Some variations too may probably have the purpose of trial and selection for a better mode of existence. It is impossible to deny that variations, which have no survival value, may be appearing and disappearing at some time or other in the course of the innumerable generations an organism goes through even in the course of twenty-four hours, giving birth to countless populations. Again, the purpose of the variability of the immunological characters of bacteria still remains a mystifying problem, while to us the subject is of profound practical importance. The relative instability of some of the bacterial antigens, the apparently fortuitous nature of their fluctuations, *e.g.* the loss or gain of the H antigen in the H-O variation or the facile alteration of the specific and non-specific flagellar antigens of the diphasic *Salmonellas*, and the random way in which antigens are distributed in the microbial populations are all points that rouse doubts whether there is any fundamental biological significance or design associated with the immunological properties of bacteria. The antigenic property of bacteria is relative and has no existence apart from the conception of parasite-host association. There is the possibility that all these variant changes represent trials in the process of evolving necessary and suitable adjustments towards a perfectly harmonious living with the natural environment, that is, a slow process of transformation from antagonism to symbiosis. They also incidentally serve to show the wide flexibility of bacterial form and function.

Bacterial Dissociation. Bacterial dissociation is a particular type of bacterial variation characterised outwardly by certain striking changes in the type of colony formed on solid media. Early workers had called attention to the appearance of different types of colonies when working with the same bacterial culture. Baerthlein observed the phenomenon in 1918; he also showed that a variety of bacteria exhibited marked colony variations. Fresh interest was roused in the subject by the work of Arkwright on the variability of enteric bacteria in 1921 and of de Kruif in

the same year on variations occurring in *Past. lepiseptica.* The latter called this kind of variation *bacterial* or *microbic dissociation.* Although dissociative variation was first studied with reference to colony characters, yet even before the recent resurgence of interest in the subject attention had been called to its intimate relation to changes in other specific characters as well. And now in the concept of microbic dissociation are included a number of important correlated variant changes.

When the ordinary culture of an organism, even a single cell culture, is plated on an agar medium, two main types of colonies develop; the smooth (S) and the rough (R). The smooth colonies are round, convex, moist and shining, while the rough ones are somewhat larger, irregular, flattened, dry, granular or wrinkled. The smooth form is regarded as the parent or normal type and the rough as the variant. Hence, the change is indicated as the smooth to rough, or S–R, variation. As a rule, organisms freshly isolated from lesions grow in smooth colonies and dissociation becomes increasingly evident with the age of the culture. Between these main types of colonies, intermediate types, partaking of the features of both S and R varieties, may also be seen. This probably indicates that the transition from the smooth to rough is a gradual process passing through several intermediate stages. When once the change has started, it goes on, though slowly, to completion and the cell structure undergoes fundamental changes in course of time with the result that the ultimate variant has lost many of the specific features of the modal cell. A stock laboratory culture may, therefore, contain organisms in different stages of transition and sometimes only the final rough phase product.

The S–R transformation may be hastened by certain artificial procedures. For example, the addition of lithium chloride or anti-S serum to the medium employed for culture of the organism hastens the process. So also the action of bacteriophage. For example, rough variants of *Bact. typhosum* containing neither Vi nor O antigen can be produced by subjecting the smooth strains to the action of bacteriophage.

The S–R transformation is easy, but the R–S reversion is relatively difficult, the R variants displaying little tendency to return to the original smooth form. This has led to the belief that the rough phase culture is much more stable than the smooth

phase culture. This relative stability of the R form would also seem to indicate that in its nature the S–R change is not of the category of the fluctuating type of variation which readily swings to and fro. Though difficult, the backward process may, nevertheless, be induced artificially in most instances by cultivating the R form in the presence of anti-R serum. Animal passage may likewise bring about the R–S reversal. This reversibility, albeit difficult, as well as the gradualness of change differentiates the S–R dissociation from mutation.

The general pattern of the variability of the colony types described above has, however, certain exceptions. For example, freshly isolated haemolytic streptococci form rough colonies. These are regarded as the normal type and when variation occurs the alteration is from the rough to the smooth type of colonies. Again, the colonies formed by the freshly isolated virulent anthrax bacillus are of the rough type, but with the supervention of variation the change is towards a smooth form which consists of less virulent or avirulent bacilli. Thus, the usual type of variation in certain cases may be in the R–S direction; nevertheless, the change is essentially of the same kind as the S–R type. There is evidence to show that the difference is, in all probability, due to a difference in the nature of the surface antigen, that is the virulent antigen, which is protein in these species instead of polysaccharide.

It has been mentioned above that variation in the colony type is not always an isolated phenomenon but may be closely correlated with a number of other changes, some of them deep seated. Many of the important specific characters appear in some way linked to a particular colony type and when the latter undergoes alteration the former too inevitably suffer the same fate.

Changes in morphology and mode of cell division may accompany S–R transformation. For some time after division of the rough variant cell, the daughter cells tend to remain adherent end to end instead of separating off and coming to lie side by side as in the case of the smooth form. The formation of short chains with sharp bends at the points of division is the result. They probably account, in part, for the granularity of the rough colony.

While growing in fluid media, the smooth form gives rise to uniform clouding, whereas the rough form tends to sediment,

leaving the supernatant fluid clear. The suspension stability exhibited by the smooth type in physiological salt solution is lost in the R form which, in consequence, undergoes spontaneous clumping. The flagellate species usually turn aflagellate, though rarely they remain motile, giving rise to rough motile variants. Similarly, the capsulated species lose the power of capsule formation. This appendage, usually polysaccharide in nature, confers type specificity to the organism; its loss during the S–R transition, therefore, inevitably results in the loss of immunological specificity and the variant product becomes group-specific, containing only a somatic antigen common to all the types of the species — group antigen. An example of this is the conversion of a type-specific pneumococcus to the group-specific variant. The characteristic surface antigen of the normal smooth form of most species is a polysaccharide, which is also mostly responsible for virulence During S–R variation this is lost, exposing a deeper seated rough somatic antigen common to the group and composed of a different kind of polysaccharide and protein. The smooth parent strains, while agglutinating specifically in the presence of their immune sera, are not agglutinated by an antiserum prepared against the rough somatic antigen, which reflects the immunological change engendered during variation. The loss of capsule or the smooth somatic antigen in the case of non-capsulated species causes a relative increase in the superficial lipoid content, transforming the organism from a hydrophilic into a hydrophobic state. This is the explanation of the autoagglutinability of the R variants in fluid cultures and in salt solutions referred to above. Closely associated with the alterations in the antigenic make-up is a diminution or loss of virulence. The immunological response of an organism is inextricably bound up with its antigenic structure. Hence, modification in the latter inevitably leads to changes in the former as well. Thus, bacterial dissociation implies also profound changes in the antigenic constitution, virulence and immunological states of bacteria. Other changes are a reduced biochemical activity and a reduced sensitivity to the lytic action of bacteriophage. The final product is thus a more hardy individual with many of the specific features altered beyond easy recognition. All those correlated changes involved in smooth to rough variation may be summed up as follows:

1. Roughness or granularity of colonies.
2. Instability in fluid culture and in physiological salt solutions.
3. Loss of capsule or very often flagella.
4. Loss of the characteristic smooth surface antigen.
5. Loss or diminution of virulence.
6. Reduction in the biochemical activity.
7. Loss of sensitivity to the lytic action of bacteriophage.

From the foregoing it is evident that the alteration in the colony type is but the superficial mark of a series of associated character changes having profound biological significance. They are apparently different manifestations of a single fundamental change, *viz.* the loss of the capacity to synthesise a particular chemical component of the bacterial cell.

The intimate nature of microbic dissociation is not clear. Whether the rough culture is a degraded variant of the smooth prototype, as regarded by many bacteriologists, or it is only one of the normal phases of the bacterial life history, as held by others, remains still unsettled. The S–R variation does not appear to be of the category of fluctuating variation which readily moves to and fro. Its gradualness of change and reversibility distinguish it from mutation. Environment undoubtedly does play a part in its causation, but how far it is responsible for or what other forces are also operative in its production, are problems which yet await solution. It has been suggested that the R form is a more saprophytic form, but that it gains during residence in the animal tissues certain new properties correlated with virulence, which are again lost when cultivated in artificial media. Much attention has not been paid to this aspect of the question; if it is proved, the S–R variation is a mere reversion to the natural state of the organism.

A different type of colony variation is what is known as the H – O variation occurring among the flagellated bacteria. It was originally described in the case of *Proteus vulgaris*. Because of its great flagellar activity, the normal form of this organism grows on agar in irregular rapidly spreading thin films. But sometimes discrete non-spreading colonies make their appearance spontaneously. This has been found to be due to the loss of flagella, which expresses itself as a colony variation from the spreading

or H (Hauch = film) type to the non-spreading discrete O (Ohne Hauch) type. The H – O variation has gained considerable importance in view of the fact that the antigens present in the flagella are entirely different from those making up the cell body. Suppression of flagella means loss of H antigen. In other words, this type of variation also involves antigenic changes in the organism, though it may not be so profound as in the case of the S–R variation. The loss of flagella also causes a physical change in the type of clumps formed during agglutination; the fluffy clumping seen when dealing with the flagellar strains is given place to a granular type of clumping with the non-flagellated strains. In case the flagellar antigen is also type-specific, then its loss deprives the O variant of any type specificity. The O form, however, does not undergo any antigenic change beyond the flagellar loss; it retains the smooth somatic or O antigen unlike in the case of the S – R variation in which the loss of the superficial O antigen is a conspicuous feature. The H – O variation is quite independent of the S – R variation.

The nature of the stimulus producing the H – O variation is unknown. This type of variation differs from the S – R variation in that the transformation occurs more readily than in the latter. But the non-motile O variant usually shows little tendency to reversion. The H – O transformation can be easily induced by artificial means, for example by growing the motile strain on agar containing 0·1 per cent. phenol. Here also the suppression of the faculty to develop flagella is prompt, but, unlike when naturally occurring, the change is readily reversible and the non-motile O variant is retransformed into the common motile form immediately it is transplanted on ordinary media. It is, therefore, a case of temporary adaptation.

A different type of flagellar variation is met with among the *Salmonellas*. The flagellar or H antigen in these organisms may exist in two immunologically distinct forms, the *specific phase* (*phase 1*) and the *group phase* (*phase 2*). Refer under *Salmonellas*. Bacterial species, whose H antigen may exist in both forms, one or other in different individuals of the same species, are termed *diphasic*. An ordinary culture of such a diphasic organism will contain representatives of both forms. On plating out they give rise to two classes of colonies which, while morphologically similar, are immunologically distinct, one class

containing exclusively individuals with specific phase antigens and the other individuals with group phase antigens. By subculturing separately from single colonies, pure phase cultures can be obtained. Cultures possessing the specific H antigen agglutinate only with the specific serum, whereas those possessing the group H antigen display considerable cross agglutination with allied organisms. The phasic cultures are very unstable, and change in either direction may occur after a few subcultures, each phase giving rise to mixed descendants. The transition in all these cases is prompt and does not have the gradualness of S – R variation. The phasic variation is, therefore, of a fluctuating type. It may be further noted that the O antigen remains unaffected during the phasic variation which occurs independent of S – R variation.

Besides the *specific-group phase variation*, the specific phase or phase 1 may itself exhibit a diphasic variation termed the *αβ phase variation*. The antigenic components of the alpha phase differ in constitution from those of the beta phase (p. 271).

A few more types of variations in the colony forms have been described. The occurrence of the G type has been dealt with in a previous section (p. 333). Another is the mucoid or M type which is believed by some observers as cyclogenic. The occurrence of it has been noted in the colon-typhoid group of organisms. It may be unrelated to the mucoid type observed in streptococci and pneumococci. The D type (dwarf colony) is yet another variant. In comparison with the R colony, the D colony is small in size but not so minute as the G colony. In S – D and D – S variations the size of the colony appears to be the only variable character in most instances. The S – D variation probably signifies a temporary arrest of the growth vigour of the component bacteria.

Transmutation of Immunological Types. The observation made in recent years that the pneumococcus can be induced to change its type is of absorbing biological interest. It was shown that the avirulent rough form of Type I pneumococcus, when injected into an animal together with a suspension of heat-killed smooth form of Type II pneumococci, is transformed into the latter type. It was further shown that any type can in this way be converted into any other type. That is, under certain conditions a rough variant may develop the capacity of synthesising the

capsular substance of any other type: the capsular substance itself has evidently the power of inducing this faculty. Later on, it was found that interchange of types can be brought about in the test tube. By cultivating the R variant of any type in the presence of an anti-R serum and a heat-killed smooth culture of any other type, the former grows into the same type as that of the smooth culture employed. The anti-R serum is not indispensable but will accelerate the change. More recently, a substance consisting mainly of desoxyribonucleic acid has been isolated from Type III pneumococci, which in minute amounts is capable of inducing metamorphosis of rough Type II into smooth Type III pneumococci. The transmutation of pneumococcal types is a case of immunological variation brought about by artificial methods. Whether such metamorphoses occur spontaneously in nature, is still a mystery.

CHAPTER XV

THE CLASSIFICATION OF BACTERIA

The classification of bacteria was in a state of chaos for a long time. As a matter of fact, even now a stable system of classification has not been evolved. Several reasons have contributed to it. Early bacteriologists were not naturalists; they were more concerned with the behaviour of bacteria in relation to disease rather than with their nature or systematic aspect. They were largely content with the broad morphological and pathogenic characters for purposes of identification and grouping of bacteria. It is now too well known that in the classification of bacteria morphology alone is a very unreliable guide; organisms widely different from a determinative stand point are often morphologically identical. Again, in the early days of bacteriology no attempt had been made to formulate any nomenclatural code for the subject: and the dependence of taxonomy on a stable system of terminology is self-evident. Till relatively recent times, the naming of bacteria was, therefore, done haphazardly with no reference to any pre-existing well established rules of nomenclature. This was also probably due to the belief entertained by early workers that bacteriology was a new biological science, requiring rules of nomenclature and classification altogether different from those of botany and zoology. Further, the absence of any agreed and stable differential criteria for the determination of genera and species also led to indiscriminate naming. Different workers often applied different names to the same organism. For example, *Clostridium welchii* went under several names: *B. aerogenes capsulatus*, *B. perfringens*, *B. phlegmonis emphysematosae* and *B. enteritidis sporogenes*. Such a situation obviously could not lend itself to order, but instead paved the way for more confusion to creep into the literature. Nowhere is this so evident as in the formation of genera and species.

For any classification to have scientific value, it should be based on a regular system of nomenclature sanctioned by inter-

national agreement. The biological sciences, botany and zoology, had already such nomenclatures. The rules and recommendations regarding terminology are in essential identical to these two disciplines. Naturally the question arose whether these rules could be brought into service for bacteriology as well. On this important point opinions differed at first among bacteriologists. But it was finally agreed that in so far as they may be applicable and appropriate the nomenclatural codes established for botany and zoology should be adopted in the naming of micro-organisms. It should be remembered that the Linnaean binomial nomenclature serves as the basis of naming in botany and zoology.

Nomenclature. The term nomenclature is applied to the naming of plants and animals. It is necessary for a correct use of names that the student should be familiar with a few simple rules of nomenclature. Each distinct kind of bacterium is called a species. To each distinct species a name is given consisting usually of two Latin words, as *Bacillus subtilis*. The first word is the name of the genus or group to which the organism belongs. It is a Latin or less commonly a Greek noun, and when used as a scientific generic name it should always be written with a capital letter. It may be masculine, feminine or neuter in gender. Examples are *Bacillus* (masculine) a small rod; *Sarcina* (feminine) a packet or bundle; *Clostridium* (neuter) a spindle. The second word in the scientific name is a specific epithet. It is not capitalised except that certain authors capitalise specific names derived from proper nouns. The specific name is an adjective or a noun and generally agrees with the generic name in gender. The word albus, which means white, is masculine gender, the feminine and neuter being alba and album respectively. The correct naming of white *Sarcina*, therefore, is *Sarcina alba* and not *Sarcina albus* or *Sarcina album*.

The generic and specific names are often explanatory of the shape or arrangement of the cells as in *Bacillus* or *Staphylococcus*, after some person as in *Brucella* or *welchii*, after some locality as in *melitensis* or *newcastle*, descriptive of some property as in *aureus* or *pyogenes* or denoting some disease as in *tuberculosis* or *leprae*.

When a name is added after the systematic name of the species, for instance *Bacillus subtilis* Cohn, it merely indicates the author who first published the name and not the person who

first discovered or described the species. The use of trinomial and quadrinomial names is uncommon in bacteriology. Casual or common names, like gonococcus, tubercle bacillus, typhoid bacillus, plague bacillus and the like, are very freely used in preference to more formal systematic names.

A generic name can be applied to only one genus. If more than one name has been applied in the past to the same genus or species the law of priority requires that the oldest published one should be recognised as its correct name. Schaudinn, in 1905, called the newly discovered organism of syphilis *Spirochaeta pallida.* It was then pointed out that the name *Spirochaeta* had already been applied by Ehrenberg in 1834 to a different organism, thus making it untenable to use it for the new group. The generic name *Spironema* was later on proposed to it. It was again found that this name also had been previously used to denote certain groups of higher forms of life and consequently it was not permissible to use it. *Microspironema* was then proposed as a generic designation. But Schaudinn himself had by this time published the name *Treponema* for the new genus, and so following the law of priority the valid name of the organism of syphilis became *Treponema pallidum.*

Taxonomy. Taxonomy is that branch of biology that deals with classification or the orderly arrangement of plants and animals. A suitable nomenclature is indispensable for its development.

Species (pleural, species) represents a group of individuals possessing identity of behaviour and form. Such groups of like individuals, or species, constitute the unit of classification. The similarities and resemblances marking off the groups should exhibit a certain amount of constancy when tested over long periods and wide fields. Again, the common characteristics should be based on natural attributes as opposed to artificial ones. The original culture employed in the study of the specific group characters for the purpose of establishing the species is called the *type culture.*

Variations such as the smooth (S) and rough (R) types or the filterable forms called the G types evinced by certain organisms are not sufficiently constant or pronounced for constituting separate species. They are but random deviations occurring during the bacterial life, the salient specific features remaining

unaffected. Terms like "variety," "race," "strain" and "type" are employed to denote some minor differences in the properties of the species. Such minor differences, though natural, are considered too trivial to form bases for creating separate species. Examples of these are types of pneumococci, types of tubercle bacilli, asporogenic variety of *B. subtilis* and the like. Without doubt, the position regarding these minor subdivisions will have to be revised when we come to know more completely about such minor characteristics.

Genus. On further examination, a number of species will reveal some similarity of features between them, but the extent of it will be less than what exists inside any such individual group. Such correlated species are grouped together under a larger category—the genus. Again, those common generic features must be natural and constant. As the groups go on widening or more comprehensive the common group characters go on narrowing. A genus may have one or more species in it. Analogous to the type culture there is the *type species*, *i.e.* the first species studied and pronounced as belonging to a genus. Other species included in the genus will show constant and close relationship with the type species.

The absence of any clear definition of the concept of genus is indeed a great hindrance in the progress of stabilising classification. On the question of the degree of similarity that should exist among the species of a group to entitle them to constitute the same genus there is no general agreement. In no small degree has this contributed to the confusion in terminology. Added to this is the difficulty due to the fewness of genera that have received official recognition. Recent years have witnessed the creation of several new genera, but they have not received international agreement. That is mainly because the differentiating criteria forming the basis of such creation have not appeared sufficiently weighty. In spite of official non-recognition, many of them have received international usage. The names *Salmonella* and *Brucella* illustrate this. On the other hand, several suggested generic names, though recognised by certain systematists, have failed to gain any wide currency. Examples of such are *Alcaligenes*, *Eberthella*, *Shigella* and *Escherichia*. In the absence of any agreed scheme different bacteriologists follow different schemes. The typhoid bacillus is mentioned variously as *Salmonella typhi*,

Eberthella typhosa, *Bacillus typhosus* and *Bacterium typhosum*. The inevitable result of all these is a lack of uniformity and a pleothora of confusion. In the interest of taxonomic stability it is highly desirable to use, as far as reasonably possible, only such names as have received the largest measure of agreement among systematists.

A group of related genera constitutes a family. The name of the family ends in *-aceae* (added to the root) as in *Bacillaceae* which contains the two genera *Bacillus* and *Clostridium*. The word denoting family is regarded as pleural. Sometimes families are subdivided into subfamilies, these into tribes and these finally into genera. A group of related families constitutes an order. The word denoting order usually ends in *-ales* as in *Eubacteriales*. The term class connotes a group of related orders, *e.g. Schizomycetes*. The terms phylum and kingdom are still more comprehensive groups.

Bacteria are difficult to classify. Their simplicity of structure is in no small measure responsible for this. Due to this simplicity, morphology, the almost exclusive guide in the classification of higher forms of life, is inadequate for the classification of bacteria. For this, therefore, physiological characters, which are regarded only as of minor taxonomic importance among higher forms of life, are also depended upon in addition to morphology. Due importance, no doubt, is accorded to the latter. In fact, morphology forms the basis of primary subdivisions of micro-organisms. But characters like metabolic activities, pathogenic properties and antigenic structure find valuable application in the systematisation of bacteria. It should, however, be remembered that there are still many wide gaps in our knowledge of bacterial physiology. Only imperfectly do we know about the significance of many features which have been suggested as criteria for differentiation. The question of validity of accepting them as important for classificatory purposes has naturally led to disagreement among bacteriologists. Considering these many difficulties, it is not surprising that a final unassailable system of classification has not been reached.

No fewer than sixty to seventy schemes of classification of bacteria have been proposed. The very multiplicity of these is indicative of their unsatisfactory nature. They have been summarised by Buchanan, Breed and other systematists. Most of

TABLE VIII

Systematic Position of Bacteria

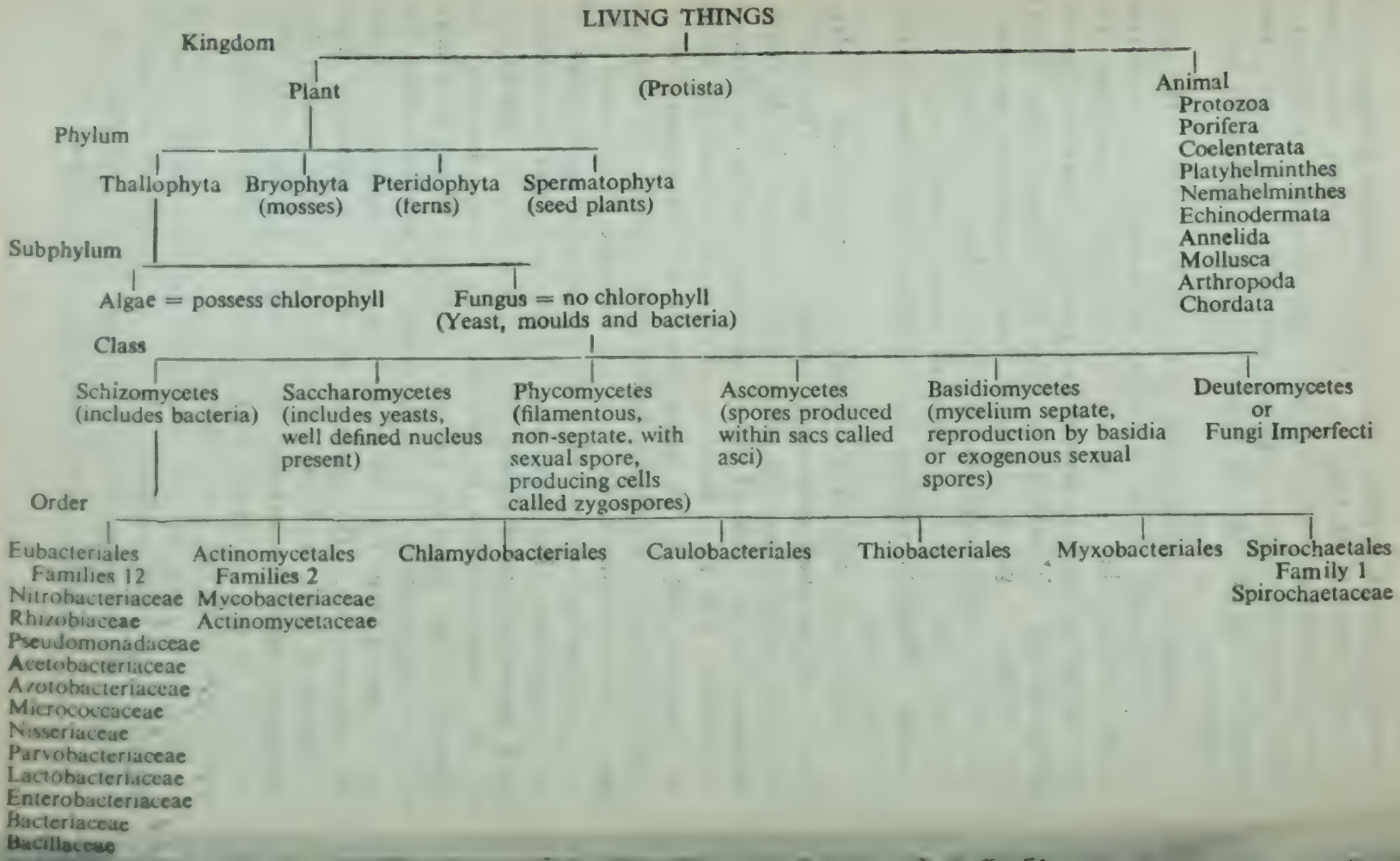


TABLE IX

Class: Schizomycetes

[*Abstract from Bergey's Manual* (1939) *with certain modifications*]

Order	Family	Genus	
Eubacteriales	Nitrobacteriaceae	Nitrobacter	
		Nitrosomonas	
		Nitrosococcus	
	Rhizobiaceae	Rhizobium	
	Pseudomonadaceae	Pseudomonas	
		Vibrio	
		Spirilla	
	Acetobacteriaceae	Acetobacter	
	Azotobacteriaceae	Azotobacter	
	Micrococcaceae	Micrococcus	*Staphylococcus aureus*
		Staphylococcus	*Staphylococcus albus*
		Sarcina	*Staphylococcus citreus*
		Rhodococcus	
		Diplococcus	
		Streptococcus	
		Leuconostoc	
	Neisseriaceae	Neisseria	
		Veillonella	
	Parvobacteriaceae	Pasteurella	
		Pfeifferella	
		Brucella	
		Haemophilus	
		Dialister	(Pneumosintes group)
	Lactobacteriaceae	Lactobacillus	(Acidophilus group)
	Enterobacteriaceae	Chromobacterium	
		Achromobacterium	
		Zopfius	
		Proteus	
		Bacterium	
	Bacteriaceae	Listerella	(Monocytogenes group)
		Actinobacillus	
		Bacteroides	(Necrophorus group)
		Fusobacterium	
	Bacillaceae	Bacillus	
		Clostridium	
	Mycobacteriaceae	Corynebacterium	
		Mycobacterium	
Actinomycetale	Actinomycetaceae	Leptotrichia	
		Erysipelothrix	
		Actinomyces	
Spirochaetales	Spirochaetaceae	Spirochaeta	
		Saprospira	
		Cristispira	
		Treponema	
		Leptospira	
		Rickettsia	Systematic position
		Virus	not yet defined.

them have now only historic value. None of these schemes has received international sanction. Unfortunately, therefore, the selection of a system still remains a matter of individual preference. The classification that is widely used at the present time, though with amendments, is the one recommended by a Committee (1920) of the Society of American Bacteriologists. The labours of this Committee have created much order where utter chaos reigned. None of the existing systems has any claim to finality. A stable classification is still in the process of evolution. With advance of knowledge what has been codified is bound to undergo considerable changes.

The common mode of classifying the pathogenic micro-organisms into the Gram-positive and Gram-negative groups has not much taxonomic significance. Nevertheless, there is much to commend it as it serves as a simple and useful guide for differentiation.

All forms of life are brought under two broad groups, the plant kingdom and the animal kingdom. Below or between these large divisions there still exist what are apparently living things but possessing no distinctive features of either of these kingdoms. They are very primitive forms, extremely minute in size and undifferentiated in structure. To include all these lowest and undefined forms the term *Protista* has been suggested by Haeckel. For many reasons, including the absence of any definite demarcating line between it and the animal and plant kingdoms, this suggestion of creating a new group has not been accepted.

Bacteria are among the simplest and the lowest forms of life and are provisionally placed in the plant kingdom parallel in position with protozoa, the lowest forms in the scale of animal life. Not that bacteria exhibit the plant characters in toto; on the other hand, they show significant departures in this respect while having certain characters in common with animals. In the absence of chlorophyll, in the possession of organs of locomotion by many and in certain complex food requirements of others, bacteria differ from the higher forms of plant life and resemble animals. Thus, a sharp differentiation is not always possible between some of the lowest forms of the two great groups. They merge into one another at certain points. Though the group comprises a diversity of developmental forms, yet at one end the actinomycetes are closely akin to the fungi and at the

other the spirochaetes to the protozoa. Hence, bacteria would appear as a connecting link between the vegetable and animal kingdoms.

The plant kingdom is subdivided into four phyla—*Thallophyta*, *Bryophyta*, *Pteridophyta* and *Spermatophyta*. Thallophyta, or plants with no differentiation into root, stem and branch, includes bacteria. *Thallophyta* is split up into two subphyla—*Algae* containing chlorophyll and *Fungus* having no chlorophyll. The latter is further divided into several classes, and of these the class *Schizomycetes*, or the fission fungus, includes the bacteria. So far seven fairly well defined orders have been recognised in this class. Each of these has been further subdivided into families, tribes, genera and species (refer Table VIII). Among the orders, the *Eubacteriales*, which form the largest and the most heterogeneous group, are also the most primitive and the least differentiated of them all. The orders *Eubacteriales*, *Actinomycetales* and *Spirochaetales* contain all the pathogenic micro-organisms (Table IX).

The systematic positions of the rickettsia (some species of which cause typhus) and the virus, both of comparatively recent discovery, remain yet undetermined. They are provisionally included under bacteria. The study of the lowest forms of life, embracing bacteria, fungi and protozoa, all too minute to observe without a microscope, constitutes microbiology. Bacteriology thus forms a part of microbiology.

CHAPTER XVI

STAPHYLOCOCCUS, MICROCOCCUS AND SARCINA

The staphylococci are the commonest pyogenic organisms. Koch and Pasteur had both observed them in pus, but it was Ogston (1881) who demonstrated their constant association with abscesses. He also proposed the name *Staphylococcus* to indicate the group. Their causal relationship to localised suppurative conditions, suppuration of wounds and to osteomyelitis was established by the detailed studies of Rosenbach (1884). He also was the first to grow them in pure culture. He obtained two kinds of staphylococci, yielding growths of different colours, and named them *Staphylococcus pyogenes aureus* and *Staphylococcus pyogenes albus*, according to the colour. In the following year Passet discovered a new variety which produced an yellow pigment; he called it *Staphylococcus pyogenes citreus*. Since then, several new species have been described, but their validity is open to serious question.

The staphylococci are Gram-positive cocci arranged in irregular groups simulating clusters of grapes. Hence the name. The irregularity in the arrangement is due to the fact that the cell division in successive generations takes place in all directions with no fixity in the planes of division. The characteristic grouping is most evident in pus and least in fluid cultures. Single cocci, paired ones, tetrads and short chains of three or four elements are also encountered. Practically all cocci are non-motile.

From the colour of the growth three species of staphylococci have been defined: *Staphylococcus aureus*, *Staphylococcus albus* and *Staphylococcus citreus*. The classification of the staphylococcus still rests on pigment production. This is unsatisfactory. As pointed out elsewhere, pigment production is inconstant, depending upon a variety of attendant conditions. The nature of pigment is also not a reliable index of pathogenicity. Again, no biochemical tests serve as sure criteria for the differentiation of

species. Serological procedures, like the agglutination and precipitation tests, have been tried, but they have not been accepted as a routine measure for identification. Other methods of classification have been suggested. One of them is based on the capacity to elaborate coagulase. The pathogenic staphylococci have been shown to be characterised by coagulase production. Other characteristics found correlated with this are the production of haemolysin, fermentation of mannite and liquefaction of gelatin. Among the coagulase-positive strains are most of the *aureus* and some of the *albus* types. *Citreus* strains are inert in this respect. It has been, therefore, suggested that the designation *Staph. aureus* should be applied for those forming coagulase and the rest be lumped together as saprophytes.

Staphylococcus Aureus Rosenbach (aureus = golden). Habitat. This organism is present on the normal skin and mucous membranes, in dust and often in cow's milk as a contaminant. It is mostly concerned in the production of suppurative lesions and is found in them.

Morphology and Staining. They are small spherical cells, ranging between 0·8 and 1·0 μ in diameter. Probably the *albus* strains are slightly larger than the *aureus* strains. In cultures on solid media and in pus, the cocci are massed in irregular clusters; in broth culture the grouping is not characteristic and solitary cocci, paired ones, tetrads and short chains are quite common. They are non-motile, non-capsulated. They stain readily with the usual coal-tar dyes and are strongly Gram-positive and not acid-fast. Irregularity of staining is a common feature of old cultures and organisms in pus, especially the phagocytosed ones.

Growth Requirements. The staphylococcus is not fastidious in its food requirements; it grows readily on all ordinary media. It is an aerobe as well as a facultative anaerobe. The optimum temperature is 37° C., but growth occurs between 12° and 45° C. The optimum pH is between 7·4 and 7·6 Toxins are best produced in certain special media (Walbum's) and under a high (20–25 per cent.) carbon dioxide tension.

Cultural Characters. The growth on solid media is abundant, opaque, smooth and coloured a rich golden yellow. The colonies on agar appear as discrete, circular, low convex, glistening, coloured discs, 1–2 mm. in diameter after twenty-four hours. On blood agar plate the colonies are similar in appearance but larger

and surrounded by a clear zone of haemolysis (β type). There is uniform turbidity when grown in broth. No pigment production is noticed in fluid cultures.

Biochemical Reactions. Various carbohydrates, such as glucose, lactose, saccharose, mannite and maltose, are fermented with the production of acid. Gas is never produced. Raffinose, salicin and inulin are not attacked. Mannite fermentation is characteristic of practically all *aureus* strains.

A specific enzyme, *gelatinase*, is formed and so gelatin is liquefied during growth. In milk both acid and clot are produced; the latter may or may not be digested. The organism produces a golden yellow pigment, best on potato and at 22° C. With continued cultivation colour production is reduced or even lost. The pigment is not toxic; it belongs to the carotenoid group; its function is not known. A filterable haemolysin is formed, which is most active on rabbit's erythrocytes.

Resistance. Staphylococci are rather hardy organisms. They are destroyed by heat at 60° C. in one hour and by 2 per cent. phenol in fifteen minutes. Desiccation is resisted for long; in old cultures the organism retains its viability for many days and even months. The organism is very sensitive to various organic dyes; for example, brilliant green in a dilution of 1 : 10,000,000 is destructive to it. The antibiotic penicillin is lethal to it even in much higher dilutions.

Toxin Production. Under suitable conditions of growth, most of the *aureus* strains elaborate certain soluble toxins, *viz.* haemolysin, acute killing factor, necrotoxin or dermotoxin, and leucocidin. In addition to these, a number of other toxic factors have been described: coagulase or plasma coagulating factor, spreading factor and enterotoxin. Some strains are also said to produce a fibrinolysin. The toxic nature of the endotoxin is not well understood.

The haemolysin consists of at least two different types: the α-lysin which acts both on rabbit and sheep red cells, causing rapid lysis at 37° C., and the β-lysin which acts only on the sheep cells, causing lysis only at ordinary temperature but after a preliminary incubation—the so-called "hot-cold" lysis. A third haemolysin, the γ-lysin, has also been described. These haemolysins are antigenically different. The toxic filtrate, when injected intravenously in small doses into a rabbit, causes almost imme-

diate death in virtue of the acute killing toxin. The necrotoxin causes on intradermal inoculation acute toxic necrosis of tissues; the leucocidin exhibits a destructive action on the leucocytes. The last one has been claimed to be distinct from the other three. It is regarded by many authorities that these various factors represent only different activities of one and the same toxin under different conditions of action.

Certain strains of *Staph. aureus* produce a filterable toxin which acts as an acute irritant of the gastro-intestinal tract and for this reason called enterotoxin. It is different from the other toxins mentioned above and is heat stable. It is the cause of one form of food poisoning, milk and milk products being the usual incriminated articles of food.

Many strains of staphylococci form an enzymic factor which exhibits the capacity to coagulate blood plasma; it is called coagulase or the plasma coagulating factor (p. 215). There seems to exist a close correlation between the pathogenicity of a strain and its coagulase-producing capacity. The coagulase does not seem to be a true toxin.

Extracts made from strains of staphylococci when injected into the skin render it more permeable to invasion by staphylococci and certain other organisms. This is called the spreading factor (p. 216).

Pathogenicity. *Staph. aureus* is pathogenic to man and experimental animals, such as rabbits and to a less extent guinea-pigs and mice. It is one of the commonest human pathogens. Practically all *aureus* strains are pathogenic. Under certain conditions, staphylococci seem to penetrate the intact skin, probably through the sweat ducts or along the hair follicles. They are also constantly present on the normal skin. Hence, it is not surprising that man is subject to frequent staphylococcal affections.

Typically, the staphlococcal lesion is a localised one and the characteristic pathological reaction is the migration of leucocytes to the infected focus. *Staph. aureus* is responsible for many of the suppurative lesions in man, such as abscesses, boils and carbuncles. Invasion of blood and internal organs may also occur, leading to acute osteomyelitis, periostitis, septicaemia and pyaemia. A very fatal form of staphylococcal pneumonia has been described. Cases of intractable urinary sepsis and recurrent

nasopharyngeal catarrh are often due to *Staph. aureus.* Staphylococci may cause inflammation of any part of the body. They are also found in mixed infections and as secondary invaders, particularly in infections of the upper respiratory tract. The organism can be easily recovered from all these lesions. Staphylococcal food poisoning is discussed under food poisoning.

Of all the experimental animals, the rabbit is the most susceptible to staphylococcal infection. Subcutaneous inoculation with a virulent strain produces a localised abscess. The intravenous inoculation of 0·1 c.c. of a 24-hour broth culture kills the animal in four to eight days. On autopsy, various internal organs, particularly the kidneys and the heart, show the presence of minute abscesses.

Natural infection among domestic animals is not very common. Cows often suffer from staphylococcal mastitis. A staphylococcus seems to be responsible for the condition called botriomycosis occurring in the equine animals. It is called *Staphylococcus ascoformans.* In the lesions it occurs in colonies simulating actinomycotic granules. It resembles *Staph. aureus* in general characters.

Diagnosis. The diagnosis of staphylococcal infections is a simple matter. A Gram-stained smear of the pus reveals the irregularly arranged clusters of Gram-positive cocci. The isolation is easy and blood agar is the medium of choice. In twenty-four hours fairly large colonies with β-haemolysis in the case of all *aureus* and some *albus* strains, develop on the medium. For the identification of species a culture has to be made on agar slope. The characteristic colour, golden yellow, white or lemon yellow, develops in one or two days. But the exact type of the pigment is often difficult to determine in the early stages of growth. Other confirmatory tests are the production of haemolysin and mannite fermentation. The former can be shown by the formation of a clear zone of haemolysis round the colonies on blood agar plate or, better still, by the laking produced when 1 c.c. of a five per cent. suspension of red cells is incubated for one to two hours with 1 c.c. of a twenty-four-hour growth in broth. *Aureus* produces haemolysin and attacks mannite, *albus* may rarely form haemolysin but does not attack mannite, whereas *citreus* does neither. Gelatin liquefaction has practical difficulties in its performance (p. 97).

The Coagulase Test. The mere isolation of a staphylococcus is not always enough; determination of its pathogenicity is necessary in many cases. The production of coagulase is regarded as an index of pathogenicity. When necessary its production should also be tested for. Citrated human plasma is diluted 1 : 10 with normal saline. About 0·5 c.c. of it is mixed with three or four drops of a 24-hour broth culture of the organism and the mixture is incubated at 37° C. A saline control should also be included. Coagulation usually occurs in one hour. Rabbit plasma may also be used instead of human plasma.

Specific Treatment. Vaccines are of some use in the treatment of chronic and recurrent staphylococcal infections (p. 225). Autogenous vaccines are preferable to stock vaccines. Vaccines are prepared from a saline suspension of a 24-hour culture on agar and killed by adding 0·5 per cent. phenol. They are usually standardised to contain 1,000 million organisms per cubic centimetre. The initial dose is 100–200 million cocci, worked up to a thousand million per dose. The injections are given at intervals of 6–7 days, depending upon the reaction. The immunity so developed is antibacterial only. The stimulation of the phagocytic mechanism seems to be its virtue. Toxoid seems to give better results than vaccines. A combination of these two would seem to yield better results than either alone, as the resulting immunity would be both antibacterial and antitoxic. In acute and severe infections staphylococcal antitoxin, obtained from horses immunised with toxoid, has been tried with varying results. Large doses are required, given by the intravenous route.

The toxoid is prepared by subjecting toxin of proved potency to the action of 0·1 per cent. formalin at 37° C. for about fourteen days. By this time the detoxication is complete, but the antigenic power is not seriously affected (p. 211). Usually eight injections are given starting with 0·05 c.c. of the undiluted toxoid, gradually increasing to a maximum single dose of 0·4 cubic centimetre. Local reaction and mild general symptoms may follow, but they are not serious.

Chemotherapy. Sulphathiazole is of distinct value in the treatment of staphylococcal infections; sulphapyridine and sulphanilamide are less effective (p. 158). The discovery of

penicillin is a distinct advance on sulphonamides; penicillin is highly effective in all types of staphylococcal infections (p. 155).

Staphylococcus Albus. It resembles *aureus* morphologically and in growth characters. The *albus* type is generally less active than the *aureus* type. It is feebler than the *aureus* in its action on carbohydrates and in the production of haemolysin. The liquefaction of gelatin is not constant. Generally, it is a milder pathogen than *Stahp. aureus*. Colour production, however, is not always a sure criterion of pathogenicity; not uncommonly *albus* strains comparable in virulence to *aureus* strains are encountered. Rarely white colonies grow from a purely *aureus* strain. Such *albus* variants are very virulent. An *albus* variety normally present on the skin as a commensal has been named *Staphylococcus epidermidis*.

Staphylococcus Citreus. This is a rare variety and produces a lemon yellow pigment. Gelatin liquefaction is still less constant than in the case of *albus*; in biochemical activities it is practically inert. It appears to be non-pathogenic.

MICROCOCCUS

The genus *Micrococcus* comprises spherical cells arranged in pairs, tetrads or groups but not in grape-like clusters or chains. They are generally Gram-positive. They are biochemically weak. Some of the species form an yellowish pigment.

Micrococcus Tetragenus. They are cocci occurring in clusters of four and frequently found on the mucous membranes of the upper respiratory tract. Multiplication takes place by the individual element dividing successively in two planes at right angles, forming tetrads. In tissues each group of four is surrounded by a capsule. The organism is strongly Gram-positive.

Micrococcus tetragenus is an aerobe and facultative anaerobe. The optimum temperature for growth is 37° C. It grows well on all ordinary media, some strains rather more slowly than others. The growth on agar is white in colour, resembling that of *Staph. albus*. Gelatin is not liquefied.

It is of doubtful pathogenicity to man. Probably it is unable to invade the human tissues, except under conditions of lowered resistance. Frequently it is present in the sputum in cases of pulmonary tuberculosis, lung abscess and other inflammatory

conditions of the lungs and the respiratory tract. Very rarely it has been isolated alone from materials derived from acute abscesses, septicaemia and meningitis. It is highly pathogenic to the mouse, which marks it off from other micrococci.

The Pyogenic Bacteria. The formation of pus on the wake of acute inflammation is a characteristic feature in a number of bacterial infections. The organisms responsible for such infections are, for convenience of description, termed the pyogenic organisms. This is largely an arbitrary division. Several organisms that are not usually termed pyogenic may, however, lead to pus formation under certain conditions. For example, the typhoid or the tubercle bacillus may in certain situations produce inflammatory exudates containing a large proportion of neutrophile leucocytes. Many organisms not called pyogenic are likewise capable of producing pus under experimental conditions. True suppurative lesions clinically met with are practically all due to one or other of the pyogenic bacteria. The commonest pyogenic organisms are the staphylococcus and the streptococcus; not all species of these are pyogenic. Various other organisms are also associated with pus formation. They are the pneumococcus, gonococcus, meningococcus, *M. tetragenus*, *Ps. pyocyanea*, *Bact. coli*, *Proteus* and *Actinomyces.*

The essential changes that take place in suppuration are necrosis of tissue caused by the irritant toxin, accumulation of leucocytes, predominantly of the neutrophile type, at the focus of infection and the digestion and liquefaction of the dead material by proteolytic ferments. These ferments mainly come from the leococytes and to a lesser extent from the necrosed tissue cells and the invading organisms. They have no action on living cells. The resulting fluid product is called pus. This is the method how dead material is removed from the body. The leucocytes also, like the tissue of the inflamed part, undergo degeneration due to the action of toxins liberated by the invading bacteria and subsequent digestion by ferments. It must be borne in mind that the mere presence of leucocytes does not constitute suppuration. The other criteria also must be present. Suppuration may be circumscribed or spreading and it may or may not be associated with demonstrable clinical symptoms, but the pathological changes that take place are essentially the same.

SARCINA

The *Sarcinae* are Gram-positive cocci arranged in cubical packets due to the cell division taking place in three planes. They are not pathogenic. The aerobic ones are pigment-producers. *Sarcina ventriculi* is occasionally found in the stomach contents of patients suffering from gastric diseases.

CHAPTER XVII

STREPTOCOCCUS

The *Streptococci* constitute an important group of pyogenic cocci that grow in the form of chains. Their relation to diseases of man was first noted in the case of erysipelas. The constant presence in this disease of cocci, existing in the form of chains, was first observed by Koch (1881). In the same year Fehleisen also made the same observation: further, he succeeded in growing the organism in pure culture (1883) and called it *Streptococcus* of erysipelas. The term *Streptococcus* was first used in a generic sense by Rosenbach (1884) to describe a chain-forming coccus, which he isolated from acute abscesses in man. He gave it the binomial specific name *Streptococcus pyogenes.* Further proof of its causal relationship to suppurative lesions in man was provided by Ogston. Since then, a large number of streptococci have been isolated from human, animal and other sources.

Streptococci are spherical or ovoid cells, occurring in chains of variable length or in pairs. Typically, individual cells divide only in one direction, and the elements of the successive generations remain close together instead of separating off completely. Hence the chain formation. As a rule, they are not capsulated. Generally, they are Gram-positive and non-motile. Most species are aerobic and facultatively anaerobic; some are strictly anaerobic. Streptococci are normally present in the air, water, milk and dust; but the majority of them are non-pathogenic. Many species are found as normal parasites on animals and man, and some are highly pathogenic to both.

Classification. The classification of streptococci is far from satisfactory. Many differential criteria have been suggested from time to time, but none of them has proved satisfactory as the sole basis of classification. On the basis of oxygen requirements, an initial classification may be made into the aerobic and the anaerobic streptococci. Not much is known about the latter; they seem to constitute a relatively small group.

Attempts have been made to classify the organism on the basis of morphology. With the exception of enterococci, the differences in the morphology of the streptococcal cells do not offer any aid in classification. The size of the chain formed has been employed as a basis for differentiation. But soon it became evident that this method too is not reliable, as chain formation is a variable factor, depending upon the circumstances of growth. With any one type the length of chain formed was found not a constant feature. Even a single strain, whether virulent or not, may sometimes grow in short and sometimes in long chains, conditioned by the growth environments. Enormously long chains of the comparatively less virulent anhaemolytic type are not infrequently seen, particularly in suppurative lesions of the lung, while the very virulent haemolytic type may at times appear in short chains in purulent materials. Thus, chain formation is not a stable feature and cannot be relied on for classification. Hence, there is no justification in retaining terms like *Streptococcus longus* and *Streptococcus brevis.*

Fermentative characters have been employed as differential criteria and many schemes of classification based on them have been put forward. Although certain of these characters are of some differentiating value, for example the fermentation of mannite and aesculin by the faecal streptococcus, it is doubtful whether fermentation reactions are, on the whole, stable enough to form bases for classification.

Differentiation was sought to be established on clinical grounds, but it is unscientific and has only led to greater chaos. Names like *Streptococcus erysipelatis*, *Streptococcus scarletinae* and the like have no systematic value and should be avoided.

A number of old names are based on the source of the organnism. Such are *Streptococcus salivarius*, *Streptococcus lactis* and *Streptococcus faecalis.* These names, though not based on strictly scientific criteria, have, nevertheless, come to stay.

Bacterial Haemolysins. Many bacterial species elaborate during growth certain factors lytic on the erythrocytes of higher animals, which are, therefore, called haemolysins (p. 213). When growing on blood agar, they cause haemolytic changes in the medium immediately surrounding the colony, producing certain characteristic appearances. Some species produce complete haemolysis round their colonies, giving rise to sharply defined,

clear, colourless zones in sharp contrast to the reddish opacity of the medium. These zones vary in size with different species. No intact corpuscles are left in the zone as shown by the microscope. This is called the β type of haemolysis. Other species produce a greenish discoloration of the medium instead of complete clearing. Immediately round their colony is seen a greenish zone which is not well defined, and outside this is another narrower clearer but not discoloured zone. In the greenish zone many intact but discoloured corpuscles are seen under the microscope, but in the outer clear zone only a few cells which are never discoloured are present. This type of partial haemolysis is called the α-type. It has been suggested that the greenish discoloration may be due to the formation of a green pigment which is probably an oxidation product of haematin. Another view is that it is due to the formation of methaemoglobin. Against this is the fact that the green colour is more marked when the organism is grown on media containing heated blood.

As originally suggested by Schottmüller, the haemolytic property, as observed on blood agar, has been provisionally accepted as the basis of primary classification. Not that it is altogether satisfactory, but there is no other stable criterion so far discovered. With reference to it, all the aerobic streptococci can be brought under three groups. Those that cause complete lysis of the red cells, producing a clear zone round colonies on blood agar, are grouped as haemolytic streptococci, or the β type. They correspond to Schottmüller's *Str. haemolyticus*. Usually the term haemolytic, as used thus, merely indicates haemolysis on blood agar and it should be remembered that a filterable haemolysin has not been isolated from all streptococci haemolytic on this medium. Streptococci that bring about changes in the blood marked by the production of a greenish discoloration of the medium round the colonies, as mentioned above, are called viridans streptococci, or the α type. Schottmüller called such strains *Str. viridans*. The rest of the species are apparently inert to the red cells and present no visible changes of the blood medium during growth. They are grouped together as the anhaemolytic or the non-haemolytic streptococci, sometimes referred to as the γ type.

These groups are very broad and heterogeneous, each containing several subgroups and types with wide variations in pathogenicity, biochemical and immunological properties and in

habitat. Hence, it is highly undesirable to represent each of these groups as a single species, *Streptococcus haemolyticus*, *Streptococcus viridans* and *Streptococcus anhaemolyticus*, as is often done.

Recent attempts to classify streptococci on the basis of their antigenic differences have led to what appears to be a more reliable scheme of classification. Antigenic fractionation of the streptococcus shows a complex antigenic make-up. Three antigenic fractions have been identified: a type-specific protein "M" which is specially abundant in freshly isolated virulent strains, a group-specific carbohydrate hapten "C" which forms an integral part of the bacterial cell and a non-specific nucleoprotein "P" which is common to all strains of streptococci and also to pneumococci and staphylococci. Yet another antigen "Y", of which little is known so far, has also been recognised. In addition to these, one more antigen, designated "T" substance, has been identified recently. It is also type-specific but different from the M substance. Unlike the latter, it resists proteolytic digestion. The M substance is found only in mucoid or matt colonies, whereas the T factor is found not only in mucoid and matt but also in the avirulent glassy colonies. The T substance may occur in association with the M factor or independently of it.

Employing the precipitation reaction, Lancefield and collaborators have shown that all the known strains of the haemolytic variety fall under nine immunologically different groups. These are indicated by the letters A, B, C, D, E, F, G, H and K. Recently, three more groups, L, M and N have been added, thus making a total of twelve groups. Grouping depends upon the group-specific polysaccharide antigen C. The soluble carbohydrate C of one group is different antigenically from that of another group. That is, there are twelve different kinds of C substance, each specific to a particular group. The association between the haemolytic property and the grouping is somewhat loose. Some groups at least contain non-haemolytic strains; for example, *Str. lactis* which is uniformly non-haemolytic is assigned to Group N, as it contains a special kind (N) of group-specific polysaccharide C antigen. Again, it is important to bear in mind that the term haemolytic, as used here, refers only to the lytic phenomenon displayed on solid blood agar and does not imply the elaboration of a filterable haemolysin; no filterable haemolysin has been obtained from all haemolytic members.

This grouping is to a large extent correlated with the natural habitat of the organism. The human pathogens mostly fall under Group A, and Groups B and C mainly represent animal pathogens. The term *Streptococcus pyogenes*, first introduced by Rosenbach, corresponds to Group A; it is not synonymous with the term *Streptococcus haemolyticus* which is better avoided for reasons given above. Some of the strains falling under Groups C, F and G would seem to be feebly pathogenic to man. They are often found normally in the upper respiratory tract, mouth, intestines and the genitalia. Any one of them may sometimes be isolated alone from suppurative conditions. The other groups are not known to be definitely pathogenic to the human beings. The occurrence of Group B infection in man has been reported; organisms of this group may be found as commensals in the human vagina and throat. Haemolytic streptococci are normally found in the nasopharynx of about 20–25 per cent. of healthy individuals in the temperate regions. This appears to be too high a figure for the tropics. Groups B and C contain mostly animal pathogens. Group B includes strains isolated from cases of chronic mastitis in cattle. It contains both haemolytic and non-haemolytic strains all possessing a common group-specific polysaccharide antigen. This group corresponds to *Str. agalactiae*. Outbreaks of sore throat and scarlet fever due to haemolytic streptococci of bovine origin have been reported, transmission of infection occurring through milk from cows suffering from mastitis.

By agglutination and agglutinin-absorption tests, pathogenic haemolytic streptococci have been divided by Griffith into twenty-seven serologically distinct types. Recently, three more types have been identified, making a total of thirty types. Of these, 26 types belong to Group A. The protein component M present in any one type is peculiar to that type, and this forms the basis of differentiation between types. Any one of the 30 types may be encountered in the human lesions, but most of them are of rare occurrence. Types 1, 2, 3, 4, 6 and 8 are the common ones. The other groups are also heterogeneous and the existence of specific types in some of them has also been established. For instance, three of Griffith's types, Types 7, 20 and 21, belong to Group C and Type 15 to Group G.

Streptococcus Pyogenes Rosenbach. *Streptococcus pyogenes* is another important pyogenic organism. It causes several serious

disease entities and is responsible for many acute and chronic ailments, alone or in combination with other organisms.

Morphology and Staining. Streptococci are spherical or ovoid cocci, 0·6–1·0 μ in diameter, arranged in chains of varying length. Chains of less than eight cocci seldom occur, but on solid medium the cocci may occur in pairs. Distortion in the shape of individual elements is a frequent phenomenon, especially in old cultures. They are non-motile, non-sporing and usually non-capsulated. They are Gram-positive and non-acid-fast.

Growth Requirements. This organism is more fastidious in its food requirements than *Staph. pyogenes* and does not grow well on ordinary media; enrichment with materials like glucose, blood and serum markedly improves growth. It is aerobic and facultatively anaerobic. The temperature range is between 25° and 42° C. with an optimum of 37° C.

Cultural Characters. On agar *Streptococcus pyogenes* grows as discrete, minute, pin-point, translucent, convex, slightly granular discs less than 1 mm. in diameter after twenty-four hours' incubation. On blood agar the colonies are larger and surrounded by a zone of β-haemolysis. The colonies have a granular surface with a rough appearance, especially when the strains are recently isolated. This type of colony is referred to as "matt". There seems to exist some correlation between virulence and the matty appearance. On subculturing, the rough and matty strain dissociates into rough and smooth colonies. The smooth "glossy" variants will have lost the virulence. Intermediate rough avirulent variants have also been described. The R–S variation is found to be associated with the loss of the specific protein component M of the organism (p. 345). A "mucoid" type of colony consisting of virulent forms may also occur.

In fluid media *Str. pyogenes* usually grows in long tangled chains which remain at the bottom as a granular deposit, leaving the supernatant fluid clear. Sometimes growth may take place in short chains, causing general turbidity of the medium. In any case, there is a close association between the type of growth in a fluid medium and the length of chain formation, the shorter the chain formed the greater the turbidity.

Biochemical Activity. Many sugars, like glucose, lactose, maltose, salicin and trehalose, are fermented with the production

of acid but not gas; inulin, sorbite and raffinose are not fermented; mannite may be occasionally attacked. *Str. pyogenes* does not hydrolyse sodium hippurate. Litmus milk is rendered acid but not sufficiently to cause clotting. Indole is not produced and gelatin is not liquefied. A filterable haemolysin is formed.

The haemolysin is produced early during growth. On blood agar it causes the β type of haemolysis. Both superficial and deep colonies produce haemolytic clearing. Some of the viridans type occasionally show a complete pseudoclearing on surface seeding, but this does not occur with deep colonies. For this reason shake cultures are far more reliable for demonstrating the presence of haemolysin. For uniformity of results, blood from the same source should be used in all experiments. Horse blood is preferable. A still better test for the demonstration of haemolysin is provided by the action of a fluid culture on a suspension of red cells in physiological salt solution. By this method lysis is produced only by the beta strains (p. 213).

Resistance. *Streptococcus pyogenes* is a more delicate organism than the staphylococcus. It dies off soon in culture but survives for a longer time if kept at a low temperature. Grown in Robertson's meat medium and kept at 0° C., the organism remains alive for a very long time. Cultures may also be preserved by the lyophilic method (p. 137). Heat at 55° C. destroys the organism in 30 minutes. Bile does not dissolve the organism but inhibits its growth.

Toxin Production. In addition to a powerful endotoxin, *Str. pyogenes* produces under suitable conditions a number of soluble toxins, *viz.* haemolysin, leucocidin, fibrinolysin and the erythrogenic toxin. Filtrates from culture likewise show the presence of a spreading factor, also termed the diffusion factor. Here again, as was remarked in the case of *Staph. aureus*, some of the toxic factors may be different entities or may represent different effects of a single toxin.

As noted before, the haemolysin is produced early during growth. What part it plays in the pathogenesis is not clear. There is evidence to show that two kinds of haemolysins are produced in suitable fluid cultures: one is oxygen-sensitive but capable of reactivation by reduction—the O type; the other is oxygen-stable but sensitive to acid and heat—the S type. They seem to be immunologically distinct. The leucocidin is also formed

early during growth (p. 214). Like the haemolysin, the leucocidin is also antigenic. It is more resistant than the haemolysin but is destroyed by heat at 60° C. in 30 minutes. Different strains may produce varying amounts of leucocidin. In vivo as well as in vitro it has a destructive action on polymorphonuclear leucocytes but apparently none on macrophages. The fibrinolysin is markedly heat-resistant (p. 215). It seems to have a selective action; it dissolves human fibrin and liquefies clotted human plasma (in 10 to 45 minutes) but does not act on rabbit fibrin. Fibrinolysis may probably explain the relative absence of fibrin in inflammatory exudates produced by haemolytic streptococci. Fibrinolysin is also produced by the virulent strains of Groups C and G.

The erythrogenic toxin is a real exotoxin, filterable, antigenic and flocculated by antitoxic sera. There is some evidence to show that it is not a simple substance but composed of several components. Unlike most extracellular toxins, it is relatively heat-resistant, requiring for its complete inactivation a temperature of 96° C. for 45 minutes. The detoxicating action of formalin on the erythrogenic toxin is not constant, and for this reason it is difficult to prepare a suitable toxoid. There is ample evidence in support of *Str. pyogenes* as the causative agent of scarlet fever, and it is now almost certain that the clinical manifestations of this disease including the rash are due to the action of the erythrogenic toxin. The infection is practically confined to the throat where the organism multiplies and liberates the toxin which through absorption into the circulation reaches the susceptible tissues. The erythrogenic toxin is also referred to as the scarletinal, rash producing or the Dick toxin.

Suitable strains of streptococci belonging to Group A are grown in Hartley's broth for forty-eight hours at 37° C. The cultures are then centrifuged at high speed and the clear supernatant fluid decanted. This then is filtered through any of the usual bacterial filters—Berkefeld or Seitz. The filtrate forms the toxin. As a preservative 0·5 per cent. phenol is added. The toxin so prepared is usually concentrated, and for use appropriate dilution with sterile physiological salt solution has to be made. Usually it is carried out to the extent that 0·2 c.c. of the diluted toxin contains one skin test dose (see below). The toxin can also be concentrated and partially purified.

Laboratory animals, with the possible exception of rabbits, do not react to this toxin. Hence, animal experiments have not so far proved to be a satisfactory method for the standardisation of the toxin. Man is very susceptible to it, and intradermal tests on susceptible individuals are employed for standardising the toxin as well as the antitoxin. The toxic filtrate, when suitably diluted and injected intracutaneously, produces in a few hours a local erythematous patch which reaches its maximum within twenty-four hours. The minimum amount of toxin necessary to produce an erythematous reaction of at least one centimetre in diameter within twenty-four hours after intradermal injection on a susceptible individual is taken to be the "skin test dose" (S.T.D). A "test dose" is five times the skin test dose.

Antitoxic serum is prepared by the immunisation of horses with the erythrogenic toxin (p. 256). It has some therapeutic value in scarlet fever and to a less extent in other conditions due to *Str. pyogenes*, such as erysipelas, puerperal sepsis and others. Accurate standardisation of the antitoxin is difficult due to the non-availability of susceptible laboratory animals. For this purpose neutralisation tests on susceptible human beings are resorted to in America, injecting intradermally mixtures of toxin and serum. The unit of antitoxin is ten times the smallest amount of antitoxin required to neutralise a test dose of toxin. That is, one unit of antitoxin should just neutralise fifty S.T.D of toxin. An initial therapeutic dose of 6,000 units has been recommended. A concentrated and refined serum, prepared as in the case of diphtheria antitoxin, is also available. It may be given in 20 c.c. doses intravenously.

Dick Test. Persons susceptible to scarlet fever can be detected by the intradermal inoculation of the erythrogenic toxin. The method is known as the Dick Test. A suitable toxic filtrate is diluted (about 1 in 1,000) and 0·2 c.c. of it, containing one S.T.D, is injected into the previously cleaned and sterilised skin of the forearm. A similar dose of the same diluted toxin, but which has been previously detoxicated by heating at 100° C. for one hour, is injected in a like manner into the other forearm as a control. In positive cases an erythematous patch develops at the site of injection in six to twelve hours, attaining its maximum in twenty-four hours. A reaction is accepted as positive when the rash is not less than one centimetre in diameter. The rash

begins to fade slowly at the end of twenty-four hours. On the control arm a transient erythma may be seen following inoculation but soon disappears, leaving no redness by the time the readings are made. Pseudoreactions, consisting of erythematous changes both at the control and the test sites, are uncommon in children. A positive reaction indicates the absence of antitoxin (natural or acquired) in circulation and consequent susceptibility to scarlet fever.

A positive Dick reaction is also obtained in the early stages of scarlet fever, but thereafter the degree of reaction becomes less and less and finally negative after the third or fourth week of the disease. The negative reaction is a proof of the presence of circulating antibody to the scarletinal toxin. As the disease progresses antibodies are generated by the tissues and in surviving cases immunity is established. Hence the disappearance of the reaction. When 1 ml. of serum from a patient, convalescent from scarlet fever, is injected intradermally in an early case of scarlet fever, wtih the typical bright red rash, it causes the disappearance or blanching of the rash around the site of injection. This phenomenon is known as the *Schultz-Charlton* reaction. If the injection is repeated with serum withdrawn from a patient in the early stages of an attack, no blanching of the rash follows. That is evidence for the presence of neutralising antibodies in the convalescent serum and for their absence before an attack of scarlet fever and in the early stages of it. This reaction can also be obtained by employing the antitoxic serum. It is of help in the diagnosis of doubtful cases.

The Dick test is employed in the detection of the susceptible individuals in a community before taking specific prophylactic measures. Dick-positives can be actively immunised by the subcutaneous injection of the dilute toxin. Increasing doses are given weekly or fortnightly; starting with 500 S.T.D, the amount is gradually increased to 100,000 S.T.D in the course of 5 injections, keeping the volume of inoculum always constant at 1 ml. By this method the Dick reactors become negative and remain immune for a period of 1 to 5 years.

Pathogenicity. *Streptococcus pyogenes* is an important pathogen of man. The common laboratory animals are not markedly susceptible to it. Some strains, however, may be highly pathogenic to rabbits and mice. In bigger animals *Str. pyogenes* may rarely cause spontaneous infection. Infection

with streptococci may be generalised or localised in any part of the body and assume a wide variety of clinical forms. This organism is the primary cause of a number of conditions such as scarlet fever, erysipelas, sore throat, tonsillitis, mastoiditis, meningitis, acute bacterial endocarditis, empyema, peritonitis lymphangitis, cellulitis, abscesses, septicaemia (*e.g.* puerperal fever) and pyemia. In addition to its primary role, *Str. pyogenes* may also be present in mixed infections. It is also responsible for certain serious secondary infections such as bronchopneumonia following measles, influenza, whooping cough and others.

This diversity in the clinical manifestations may be due to variations in one or more of the three pathogenic properties: toxicity, pyogenicity and invasiveness. There is no doubt that such differences also depend upon the state of resistance of the individual. All the various toxic products are formed by all the strains, albeit to a greater or less extent, and the differences between the different strains are quantitative rather than qualitative. The strains isolated from scarlet fever cases, unlike other pyogenic strains, are producers of large amounts of the erythrogenic toxin. They show but poor invasive power and do not in most cases infiltrate beyond the throat. After getting lodgement in this situation, they multiply and liberate the erythrogenic toxin which gets into the circulation and causes the disease. In this respect the scarletinal strains resemble *C. diphtheriae*. Where pus-formation is the outstanding clinical feature, as in streptococcal empyema or meningitis, pyogenicity is the predominating property of the causative strains. What particular substance is responsible for pyogenicity, is not clear. Infections characterised by a wide infiltration of the organism into the blood and tissues, as for instance septicaemia or pyemia, are due to strains possessing a marked degree of the invasive property. Thus, the present conception is that though all the three pathogenic properties are common to all the strains of *Str. pyogenes*, individual strains exhibit considerable quantitative variations in one or more of these properties.

Scarlet Fever. The streptococcal aetiology of scarlet fever may be regarded as practically established. While any type of *Str. pyogenes* (Group A) may cause scarlet fever, the first five types seem to account for most of the cases. The type incidence may vary in different regions.

The epidemiology of scarlet fever is closely analogous to that of diphtheria. Both diseases are essentially toxaemic in nature. Following primary throat infection, the clinical manifestations start. The causative organism in either disappears rapidly during convalescence. But the pace of disappearance in scarlet fever is much slower than that in diphtheria. In both diseases a certain proportion of convalescents continues to be chronic carriers, serving to disseminate the infection. The carrier rates in both preponderate over the case rates. Different workers give different figures for the carrier rate of *Str. pyogenes*; a 4–8 per cent. rate has been given by the English workers. In doing any such investigation it is important to remember that about 20–25 per cent. of the general population in the higher latitudes harbour haemolytic streptococci in their throat. This appears to be lower in the tropics.

Infection spreads probably through droplets from cases or carriers who may be of the convalescent or chronic type. Scarlet fever is infectious from its commencement. Another mode of spread is *via* dust. *Str. pyogenes* may remain alive and virulent in the dust for weeks, especially in the dust of badly lighted sick rooms. Infection in dust is from dried particles of sputum or discharge from patients. Infected fomites may sometimes act as transmitters of infection. Desquamated scales are not regarded as capable of transmitting infection. It may be remembered that scarlet fever is essentially an intoxication. Milk may serve as another vehicle for the transport of infection, and several outbreaks of scarlet fever and sore throat epidemics with milk as the incriminating agent are on record. There is increasing evidence in support of this theory. Infection of milk in the udder of a cow as the result of streptococcal mastitis is reported to be more common than direct infection of it from the throat of a human carrier employed in the milk industry. Though mastitis is mostly due to Group B streptococci, in a few cases haemolytic streptococci, indistinguishable from *Str. pyogenes*, have been isolated. How the cow gets the infection, is not clear; it may be from human carriers.

Scarlet fever is mostly a disease of the temperate regions and is seldom seen in the tropical or subtropical countries. Children in the first year of life mostly escape; the greatest incidence is in the fifth and sixth years of life. But no age is

exempt. As a rule, one attack confers lasting immunity; but second attacks are not unknown. Most of the adult population of a community within the areas of prevalence show immunity against scarlet fever. Besides the disease itself, latent and atypical infections also contribute to this. During the last few decades the incidence of scarlet fever, its severity and mortality have all considerably declined.

Bacteriology of Acute Rheumatic Fever. Acute rheumatism has till now defied all attempts to solve its aetiology. Several theories regarding its causation are in the field. One of the earliest is the streptococcal theory. As the result of extensive investigations on man as well as on rabbits, Poynton and Pain (1900) came to the conclusion that acute rheumatic fever was due to a direct infection with a particular variety or varieties of streptococci. Since then, many workers have claimed the isolation of streptococci from a large proportion of cases of acute rheumatic fever. But in the majority of instances, the materials for investigation had been obtained at autopsy. In view of the possibility of intercurrent and agonal infections and post-mortem contamination, these findings have but slight evidential value. The issue was further clouded by the fact that in many instances of successful isolation other organisms also have been obtained. On the other hand, several attempts by competent workers have failed to obtain streptococci from the blood or exudate from cases of acute rheumatic fever, throwing considerable doubt on the streptococcal aetiology.

The streptococci, whether isolated from patients or on autopsy, have been mostly heterogeneous in character and belonging to the non-haemolytic variety, either green-producing or indifferent. With these streptococci numerous attempts have been made to reproduce the disease in rabbits. Many lesions, such as endocarditis, myocarditis and arthritis, have been produced in this animal, which closely resembled those of acute rheumatism; but in many important respects there was also divergence. There is no universal agreement that such lesions reflect a true picture of the naturally occurring human disease.

This theory of direct streptococcal infection was somewhat modified later to fit in with the diversity in the kind of streptococci claimed to have been isolated by different workers from cases of rheumatic fever. According to this view, the viridans

streptococcus is the ultimate cause of the disease; but the actual rheumatic manifestation is an allergic phenomenon produced by the organism and its toxic products. The green-producing streptococci are frequently present in the body—in the tonsils, throat, mouth and other situations—as focal infections. Experimental evidence has been adduced to show that this type of streptococcus can engender a high degree of allergy in man, and hypersensitiveness developing against streptococcal products is now an accepted phenomenon. But such hypersensitivity is not peculiar to the rheumatic subjects; it is also frequent among the non-rheumatic persons. It has been postulated that the specific allergic state manifests itself as a clinical syndrome only in individuals having a background of constitutional predisposition or diathesis (p. 317).

More recently, a direct causal role has been assigned to *Streptococcus pyogenes*. The frequent association of throat infection and acute rheumatic fever is an old well attested clinical observation. Most of the rheumatic cases give a history of sore throat and tonsillitis about two or three weeks earlier. In all these throat conditions haemolytic streptococci have been found to be the cause. Relapses of rheumatic affections are also frequently preceded by the reappearance of the organism in the throat in large numbers. But there is no positive evidence yet to support the contention that acute rheumatic fever is due to the infection of blood and tissues by this streptococcus.

Immunological studies by several workers have demonstrated the presence in the blood of rheumatic cases a significantly high content of such specific antibodies as antihaemolysin, agglutinin, complement-fixing antibody, precipitin and antifibrinolysin. For instance, Todd has found that there is a marked appearance of the streptococcal antihaemolysin in the blood of the patients, increasing in titre during the acute phases and falling during intervals. Others have shown the same with antifibrinolysin and with certain other antibodies. But the demonstration of circulating antibodies against an organism like *Str. pyogenes* with its notoriously frequent association with a multiplicity of subacute and chronic affections is not the same thing as establishing the organism as the cause of rheumatic fever. No doubt, such immunological findings constitute a valuable piece of indirect evidence.

Additional support to the above theory has been tought by attempting to isolate the organism by improved cultural technique. By such methods the recovery of *Str. pyogenes* from the heart valves and pericardium has been claimed in a high proportion of cases. Against this is the fact that others, following the same technique, have consistently failed to obtain positive cultural results. There is also the finding that a large percentage of the haemolytic streptococci isolated from the throats of quiescent rheumatic cases belonged to the A group. How much significance is to be attached to this, is again a question of dispute.

Reviewing the whole position, it cannot be said that the case for *Str. pyogenes* has been established. No doubt, there is an imposing array of evidence, but none of them is complete or conclusive. At most we can say that the organism may have a part in the pathogenesis of acute rheumatism. But the exact role, if any, played by it in the causation of the disease is still obscure.

A virus hypothesis has also been suggested. In the hands of some workers, high-speed centrifugation of specimens of pericardial fluid from cases of acute rheumatism has yielded minute bodies resembling viruses. They have found such bodies agglutinable with the sera of patients suffering from acute rheumatic fever. These findings have not been corroborated by others, and this theory rests on even more vulnerable grounds than the streptococcal theory. Any infection will lower the resistance of the tissues, preparing the ground for the secondary invaders, and the virus obtained may have been only of the latter category. The same is also true of nutritional deficiencies; and the vitamin deficiency, suggested as the cause of acute rheumatism, may have only such a predisposing part in the aetiology.

Streptococcal Infections in Animals. Certain important diseases of animals are caused by the streptococcus. For example, strangles in horses is caused by *Streptococcus equi* and mastitis in cows by *Streptococcus agalactiae*. In addition, streptococci are also responsible for a variety of suppurative and septicaemic conditions, as in man. As noted above, the haemolytic streptococci causing infections in lower animals mostly belong to Groups B and C. Only very rarely are Group A strains seen in natura- infections of animals.

Immunity. Any immunity against streptococcal infections is generally very transient and of a low order. An exception to this

is the antitoxic immunity against scarlet fever. An attack of scarlet fever usually confers permanent protection, but second attacks may occur. The elaboration of antibodies, such as antistreptolysin, agglutinin and others, has been observed in streptococcal infections; but these do not seem to influence the course of infection. Phagocytic activity is not so marked a feature in these as in staphylococcal infections.

Viridans Streptococci. This group is also called the alpha type. It forms a heterogeneous group and contains several species. No satisfactory method of subdivision has yet been evolved, and differentiation is now based on fermentation reactions and other biochemical properties. Members of this group are found as commensals in the throat, mouth and genitalia. A type found in the mouth has been called *Streptococcus salivarius*. In morphology and staining reactions, there is very little to differentiate the green-producing streptococci from other types. Very long chains have been encountered particularly in suppurative conditions of the lungs.

The growth requirements of the viridans type are not so rigid as in the case of the haemolytic type. They are aerobic and facultatively anaerobic. While the optimum temperature for growth is 37° C., the viridans streptococcus grows over a wider range of temperature than *Str. pyogenes*. The former grows readily on ordinary media, but the addition of blood or serum definitely gives a more luxuriant growth. The type of growth on various media, either solid or liquid, has not much to differentiate the viridans from the haemolytic group, except for the profuseness of growth in the former, and what is described under *Str. pyogenes* regarding this applies to the viridans group as well. On blood agar, however, the colonies are surrounded by a zone of alpha haemolysis instead of the β-type. Some strains produce greater discoloration than others. There is partial haemolysis of the blood corpuscles around the colonies and the green discoloration seems to be due to the formation of a green pigment which may be an oxidation product of haematin (p. 370).

Viridans streptococci are more resistant to heat than *Str. pyogenes*. Most strains attack lactose, saccharose and often raffinose; inulin and mannite are only very rarely attacked. Bile does not dissolve them.

No soluble toxin has been demonstrated nor any haemolysin. The viridans type is definitely less virulent than the A group haemolytic streptococci; occasionally a highly virulent strain may be encountered. The viridans streptococcus is commonly associated with subacute endocarditis. It is present in inflammatory conditions like apical dental abscess, tonsillitis, bronchopneumonia, lung abscess, otitis media, mastoiditis, sinusitis and cholycystitis; in some of these it is frequently found alone.

Non-Haemolytic Streptococci. These are indifferent to blood. They are mostly saprophytic and usually found on man and in milk and various milk products; few are potential pathogens, displaying low pathogenicity to man and animals. Two well known types are *Streptococcus faecalis* (enterococcus) and *Streptococcus lactis.* The latter is found in milk and is responsible for the natural souring of milk. It contains the Group N, and not the Group D, polysaccharide.

Streptococcus Faecalis. In general appearance and staining properties *Str. faecalis* resembles the other types of streptococci. A diphtheroid appearance, even in young cultures, is not infrequent among them. Some strains of the faecal type resemble the pneumococcus in appearance but possess no capsule.

The growth is more profuse than that of even the green-producing streptococcus. The colonies are bigger and often confluent. Growth in fluid media gives rise to uniform turbidity with only slight deposit. No greenish discoloration is produced on blood agar media. Some strains of *Str. faecalis*, corresponding to Lancefield's Group D, cause β-haemolysis on blood agar.

Streptococcus faecalis is characterised by a greater resistance to heat than the viridans or the haemolytic type, withstanding a temperature of 60° C. for thirty minutes. Members of this group possess great vitality, and so preservation of culture is easy. Bile does not dissolve them. They are resistant to penicillin.

These streptococci attack lactose, mannite, salicin, aesculin and usually saccharose, trehalose and sorbite but not raffinose or inulin. They do not hydrolyse sodium hippurate. Bile salts do not inhibit their growth and so they grow freely on MacConkey's medium. Some strains of enterococci liquefy gelatin. Hence, they are sometimes referred to as *Streptococcus liquefaciens.*

Lancefield's D group streptococci are haemolytic strains of *Str. faecalis.* These haemolytic as well as the non-haemolytic faecal strains all share a common specific polysaccharide antigen, namely the Group D antigen. The species may be heterogeneous and divisible into several antigenic types. No filterable toxins are known.

Most strains are non-pathogenic and found as commensals in the mouth, throat, intestines or about the genitalia. Some are feebly pathogenic. They may cause infections of the genito-urinary or the intestinal tract. They are also found in association with some cases of subacute endocarditis.

Anaerobic Streptococci. Anaerobic streptococci are found normally on the female genitalia and in the intestines. Several distinct species have been defined on the basis of gas production and other biochemical reactions. Often they are much smaller in size than the aerobic types. A few strains are haemolytic and some form coal-black colonies on blood agar, but the vast majority are non-haemolytic. The production of gas in fluid culture is a marked feature with most strains and many of them emit a foul odour. These organisms are either strictly anaerobic or micro-aerophilic. Many strains are pathogenic to man. Anaerobic streptococci have been found in septic lesions and gangrenous conditions of the uro-genital tract, pelvis, intestines and lungs. Not infrequently, they cause puerperal infection and invade the blood stream.

Diagnosis. A preliminary examination of smears from the suspected material very often reveals the presence of Gram-positive cocci in chains. While this may be of some aid in the case of pus or exudates, especially when no other organism is present, it is of little value when dealing with materials from open wound, faeces, sputum and the like.

The isolation of the organism by cultural methods is the next procedure. The material may be directly streaked on blood agar plates and incubated in the ordinary way. If the anaerobic type is suspected, suitable methods for securing the necessary gas phase have to be adopted. Isolation from pus or exudates is a relatively simple affair, but in the case of throat swabs, sputum, faeces and others it is more laborious and repeated subculturing on blood agar plates may be necessary. In the investigation of urine a catheterised specimen (this is essential in the case of female patients) will minimise labour and yield more reliable

results. A preliminary incubation of the urine has been found to be of great advantage. Even the microaerophilic types may grow in it. Frequently, the aerobic ones grow in urine in twenty-four hours as isolated comma-shaped colonies suspended in a clear fluid. In making blood culture a minimum of 5 c.c. should form the inoculum. Hartley's broth or glucose broth may be employed. If specimens are likely to contain sulpha compounds, their bacteriostatic effect can be neutralised by the addition of 5 mg. per cent. of *p*-aminobenzoic acid to the medium.

The isolation of pyogenic organisms, like streptococci and staphylococci, from pus, discharges from septic wounds, urine and similar materials is fraught with no small difficulty when there is also present *Ps. pyocyanea* or *Proteus*. These organisms by their spreading nature prevent the formation of pure, discrete colonies of the former. This difficulty can be got over by the incorporation of certain selective bacteriostatic agents in the medium. Tellurite in a concentration of 1 : 50,000 inhibits pyocyaneus and the coli group of organisms. But *Proteus* requires a higher concentration, 1 : 20,000. Bile salts, to some extent, prevent the spread of these organisms and MacConkey's medium is useful for the isolation of staphylococci and streptococci other than the haemolytic type; the haemolytic type does not grow in the presence of bile salts.

Growth on blood agar reveals the type, but a more accurate method for testing the presence of haemolysin is by the action of a suitable broth culture on a saline suspension of the red corpuscles. One ml. of the culture is added to 1 ml. of a five per cent. suspension of horse corpuscles in physiological salt solution and the mixture is incubated at 37° C. for two hours. In positive cases complete lysis occurs within thirty minutes. The viridans type grows in green colonies on blood agar. Its differentiation from the pneumococcus is dealt with under the latter (p. 402).

Sometimes it may be necessary to rule out animal strains. Unlike the pyogenic streptococci, they hydrolyse sodium hippurate, ferment sorbite but not trehalose, grow on media containing 40 per cent. bile and do not digest human fibrin. Many of the bovine strains also behave like other animal strains.

The detection of haemolytic streptococci is relatively easy. But this is insufficient as all the haemolytic strains are not pathogenic. For instance, the mere recovery of haemolytic strepto-

cocci from a throat swab has very little clinical significance. As mentioned above, roughly twenty per cent. of normal individuals harbour in their nasopharynx streptococcus of the haemolytic variety, and of these only about a third are potential pathogens. The rest are harmless. In some cases, however, all that is necessary may be only the finding of haemolytic streptococci, *e.g.*, when a pure culture of the organism grows from a specimen of pus. But in the majority it is not enough and tests for pathogenicity have to be done. To determine whether a strain is pathogenic or not several methods are available, but they have not been well worked out and rendered easy of routine practice. Grouping is essential to determine pathogenicity, and it is done by the precipitation test, employing the group-specific sera and the soluble carbohydrate antigen extracted from the isolated organism. The isolated strain is grown in 20 per cent. trypsin broth and incubated at 37° C. for eighteen hours. The culture is centrifuged and the sediment treated with an appropriate amount of N/20 HCl, heating the tube in a water bath at 100° C. for ten minutes. It is then cooled and again centrifuged. The supernatant fluid is removed and neutralised with NaOH. This extract constitutes the specific carbohydrate antigen and is tested against the group-specific sera in capillary pipettes. The specific sera are obtained from rabbits by immunisation with identified strains of particular groups. Precipitation, observable by the naked eye, should occur within thirty minutes; often it does within three or four minutes.

Prophylaxis. Severe streptococcal infections are occasionally caused through post-mortem wounds. These should be carefully avoided. Normally the human nasopharynx is often infested with the Group A streptococcus, and there is plenty of evidence to show that the source of infection in puerperal sepsis is from the nasopharynx of the attendants or the patient herself. Therefore, the detection of carriers among the staff of lying-in hospitals and their isolation may help in the prophylaxis. Such procedures have been attended by beneficial results. Against scarlet fever the method of active immunisation of the susceptible individuals with the erythrogenic toxin is available. It has not been possible to prepare a reliable toxoid, as the toxin is very resistant to the action of formalin. Nor is this of great consequence as in the case of the diphtheria toxin; for, in contrast

to this toxin, the erythrogenic toxin is relatively harmless and serious reactions seldom follow the use of untreated toxin for immunisation. Before taking steps to actively immunise, the susceptibles of the community which is threatened with an epidemic are detected by the Dick test. They are then actively immunised as mentioned in a previous section (p. 378). Immunisation with the Dick toxin has been extensively practised on the nursing staff of many fever hospitals in the West with apparently good results. The protection induced in this way, it should be remembered, is only antitoxic. It is no check to the invasiveness of the organism. Even in the face of a solid immunity of this type, infection with *Str. pyogenes* may occur with subsequent spread from the primary focus. The spread of scarlet fever in hospitals and homes can be prevented by passive immunisation of the contacts; it may be of particular use in the case of debilitated children. The contacts are Dick-tested and the positives are given 5–10 c.c. of antistreptococcal serum. The effects are temporary, lasting not more than two or three weeks. In recent years, scarlet fever has become a less serious disease than it was two or three decades ago and this has reduced the importance of specific prophylactic as well as therapeutic measures.

Specific Treatment. Both antitoxic and antibacterial sera are available. Though both are of doubtful value, there is some evidence to show that the former is more potent than the latter. Antitoxic sera are indicated in conditions like scarlet fever where erythrogenic toxin is the prime factor in the pathogenesis. The administration of antitoxic serum has been claimed to be followed by a decrease both in the severity of the disease and in the incidence of complications. In the treatment of scarlet fever convalescent serum is preferred by some workers in the hope that it may be both antibacterial and antitoxic. The failure of antibacterial sera is probably due to the failure to employ all the twenty-six types in the immunisation of horses from which the serum is prepared. An antibacterial serum which is type-specific may have greater value.

In severe cases of acute infectious diseases, like malignant forms of meningococcal or pneumococcal meningitis, acute lobar pneumonia and septicaemias, chemotherapy alone may not always be sufficient. What is equally important in such conditions is the lifting of the heavy load of toxin already formed in the tissues.

And this should be done with all expedition. Theoretically at any rate, any hope of success towards this end lies only in the administration of large doses of the specific antiserum. For, no other substance is known to counteract the bacterial toxin.

Vaccines. It is extremely doubtful whether vaccines are of any use against streptococcal infection. The multiplicity of the pathogenic types is a serious handicap to those who believe in their efficacy.

Chemotherapy. Recent advances in chemotherapy have revolutionised the treatment of bacterial infections (p. 158). It may be recalled that prontosil had its first trial against streptococcal infections. The sulphonamides, particularly sulphapyridine and sulphadiazine, have proved to be of great value in the treatment of streptococcal infections. This is not true of scarlet fever, in the treatment of which these compounds have very little value. The exact mode of action of these drugs has not been fully revealed (p. 160). Penicillin too has an important place in the treatment of streptococcal infections; its value in the treatment of streptococcal septicaemia has been fully established (p. 155).

CHAPTER XVIII

DIPLOCOCCUS—THE PNEUMOCOCCUS

Diplococci are parasitic organisms which usually exist in pairs. The individual cells are somewhat elongated and lanceolate in shape and the pair is surrounded by a well developed capsule. The aerobic species are bile soluble. The only important species is *Diplococcus pneumoniae.*

Diplococcus Pneumoniae. The pneumococcus was discovered by Pasteur (1880) in the blood of rabbits which he inoculated with the saliva of a child dead of rabies. In the following years, the studies of Frankel, Weichselbaum and others established its causal relationship to lobar pneumonia. Weichselbaum named it *Diplococcus pneumoniae.*

There is still some disagreement about the taxonomic position of the pneumococcus. In view of its close relationship to the streptococci, some authors maintain that the pneumococcus is only another species of streptococcus and that there is no valid reason to assign it to a separate genus; they call it *Streptococcus pneumoniae.* Others regard that the differences between these two groups are sufficiently wide to give the pneumococcus a separate generic position.

Habitat. The normal habitat of this organism is the upper respiratory tract of man and animals. Pneumococci are present in the lesions caused by them as well as in the pathological products of their activity. Thus, they are found in large numbers in the consolidated portion of the lungs, sputum and very often in the blood in lobar pneumonia, in pus in empyema, in the cerebrospinal fluid in pneumococcal meningitis and in similar materials from other pneumococcal lesions. Outside the tissues, pneumococci may be found in the dry sputum particles mixed with dust in the rooms occupied by lobar pneumonia patients.

Morphology and Staining. The organism is oval or spherical in shape, 0·5 to 1·25 microns in its long diameter and occurring in pairs or less frequently in short chains. The outer end of each

element of a pair is pointed, giving the cell a lancet-shape. Variations in size and shape of individual cells are frequent. Elongated bacillary forms are occasionally seen. In tissues or when freshly isolated, each pair is enclosed in a single capsule. The capsule varies in thickness, being most marked in Type III pneumococcus. In subcultures capsule formation tends to be less and less marked and finally suppressed altogether. By the ordinary staining methods, the capsule is not stained but appears as a white halo surrounding the paired cocci. Special methods, such as that of Muir and Hiss, have therefore to be employed to stain the capsule. Pneumococci are non-motile, generally Gram-positive and not acid-fast. Occasional strains may show a tendency to Gram-negativity.

Growth Requirements. The organism is aerobic and facultatively anaerobic. A higher carbon dioxide tension (five per cent.) appears to be more favourable for growth. The optimum temperature is 37° C. with a range of 25°–42° C. The optimum pH is 7·8. The nutritional requirements of pneumococci are somewhat exacting. The growth on ordinary media is poor, particularly during primary culture. Enrichment of media with blood, serum or glucose improves growth.

Cultural Characters. The colonies on agar are small, transparent, greyish with entire margin and depressed centre. On blood agar the colonies are larger and surrounded by an area of greenish discoloration (alpha haemolysis) similar to that produced by the green-producing streptococcus. At first the colonies are flat and smooth with a vertical edge. Later the edge outgrows the rest of the surface. Further incubation produces overactivity in the centre and bossing there results. Type III pneumococcus, regarded as identical with *Diplococcus mucosus*, grows in mucoid or slimy colonies. In broth the pneumococcus grows producing slight turbidity with slight granular deposit.

Biochemical Activities. Some of the common sugars, such as glucose, lactose and saccharose, are attacked with the production of acid without gas. Inulin is fermented, differing in this respect from most strains of streptococci. Litmus milk is acidified and often curdled. Gelatin is not liquefied and indole is not produced.

The pneumococcus produces a soluble haemolysin which is readily destroyed by oxygen. Hence, it is best demonstrated

when the organism is grown under anaerobic conditions or in the early stage of aerobic growth. Autolysis readily occurs in fluid media, and cultures, therefore, very soon become sterile. It is due to the activity of certain intracellular ferments.

Bile Solubility. Bile salts dissolve the pneumococcus, sharply differentiating it from the streptococcus. This property is made use of in the identification of these organisms. To 5 c.c. of a broth culture 0·5 c.c. of ox bile or a 10 per cent. solution of sodium taurocholate is added; clearing of the medium due to lysis occurs within fifteen minutes at 37° C. Sodium desoxycholate solution gives more satisfactory results. The hydroxyl group contained in it is said to improve the lytic action. To 5 c.c. of a broth culture 0·1 c.c. of a 10 per cent. solution of the salt is added. The reaction of the culture should not be below pH 6·8, as the bile salts are precipitated in the presence of greater acidity. The precise mechanism of this reaction is not clear. It has been suggested that bile salts simply accelerate the autolytic process normal to this organism. Heat-killed pneumococci are not lysed by bile or its salts.

Resistance. It is a delicate organism. Heat kills it at 56° C. in 15–20 minutes. Pneumococci may remain viable in dust for a short time. They are more sensitive to the action of antiseptics than are many other organisms. It is particularly so to the action of quinine and some of its derivatives like optochin. Cultures die quickly. For preservation, subcultures have to be very frequently made (once in three or four days) or special methods have to be employed. A semi-solid agar medium enriched with fresh rabbit blood, when inoculated with the organism and kept in the refrigerator without incubation, maintains the organism for several months. Another very satisfactory method is desiccation in vacuo and preservation at a low temperature; by this the organism retains its vitality and virulence over long periods. Spleens of infected mice serve as a suitable material for this.

Antigenic Structure. Two main antigenic components have been recognised in the pneumococcus. One is a nucleoprotein and the other a complex carbohydrate. The former is associated with the body of the organism and is common to all the different types of pneumococci. The latter is the type-specific antigen and is associated with the capsule (p. 234). It is called the "specific soluble substance" ("SSS") or sometimes the capsular substance.

It is a complex polysaccharide. It behaves as a hapten or partial antigen, interacting in vitro with the specific antibodies but unable by itself to stimulate their production when inoculated into animal bodies. Virulence seems to be associated with the capsular substance. Type III which is the most virulent has also the greatest amount of the capsular substance.

Variation and Transformation of Types. The smooth to rough (S–R) variation occurs naturally in pneumococcal cultures. It is gradual, passing through intermediate colonial types. The change is associated with the loss of ability to form the characteristic capsule. The rough variant is almost non-virulent (p. 346). With the loss of the type-specific capsule, the new rough variant has naturally lost its specificity as well. It is still bile soluble, and the capacity to form a haemolysin and a leucocidin is apparently unaffected. The rough progeny contains only the common nucleoprotein antigen and will interact with the serum prepared against the R variant of any other type. In other words, the rough variants of all the types are immunologically identical. The S–R variation also seems to occur in the body, but its practical significance in infection is by no means clear. The R–S reversion is difficult to accomplish. But it is possible and has been brought about by animal passage as also by growing the R variant in the presence of anti-R serum or of heat-killed smooth pneumococci of the same type (p. 345).

Transformation of types has been accomplished by artificial procedures. The inoculation of mice subcutaneously with a living R culture of any pneumococcal type mixed with a heat-killed smooth form of any other type results in the conversion of the R form in the animal body into the particular S type used in the experiment. A rough variant of a Type II strain, for example, can be transformed into Type I or any other type. Transmutation of types can also be induced in vitro by cultivating the rough variants in the presence of a heavy suspension of heat-killed smooth pneumococci of the type to which change is desired. The addition of anti-R serum to the culture is not essential, but it will facilitate the change (p. 349).

Serological Classification. The polysaccharide hapten is not immunologically the same in all pneumococci; it is type-specific, exhibiting clearly defined immunological divergence between the polysaccharide factors of different types. The presence of the

capsular substance masks the nucleoprotein antigen and antisera prepared against the capsulated pneumococci display sharp serological differences. On the basis of this, thirty-two immunological types of *Diplococcus pneumoniae* have been recognised by specific agglutination tests. They are designated by Roman numerals. Formerly, pneumococci found in cases of lobar pneumonia were classified under three serologically distinct types, labelled I, II and III, and a heterogeneous lot called Group IV. Since then, the Group IV has been split up into twenty-nine types, possessing no serological interrelationship, bringing up the number to thirty-two. Recently, many more have been added, making a total of 75 types. Cross reactions occur between certain types, probably indicating some similarity in the constitution of their capsular antigens. The occurrence of mixed types, *i.e.* types reacting with two or more type-specific antisera, has also been reported. The serological types are culturally indistinguishable. One exception to this is the Type III pneumococcus. Due to a high capsular content, it gives a luxuriant mucoid growth and can be easily identified from the rest. Hence, Type III is sometimes called *Diplococcus mucosus.* There does not seem to be any justification for creating such a new species. Types I and II are sometimes referred to as the "epidemic" types, being responsible for more than 50 per cent. of cases of acute lobar pneumonia.

Typing of Pneumococcus. The identification of type is essential where serum therapy is contemplated. Three methods are available: the *Quellung* reaction, also called the capsular or the Neufeld reaction, the agglutination and the precipitation tests. The first one is a rapid and satisfactory method and saves much time. After satisfying that the specimen contains pneumococci by smear examination, small portions of the sputum or pus are mixed on slides with a drop of each of the type sera separately and then covered with cover glasses. The same procedure is followed when the material is a suspension of pneumococci. Examination by the 1/12 objective under slightly restricted light shows a sharply defined, swollen, ground glass appearance of the capsule in the presence of the homologous serum. A drop of methylene blue introduced in the preparation improves the appearance by giving sharper contrast to the transparent unstained capsule against a blue background. Preparations with hetero-

geneous sera show no capsular swelling. The reaction is rapid, the change occurring in a few minutes. In view of the large number of types, a preliminary testing with combinations of the type sera will greatly facilitate work. This is followed by testing with individual sera constituting the positive pool. How best to combine the sera, is determined by the type incidence in the locality. The American workers recommend grouping as follows: (*a*) 1, 2, 7; (*b*) 3, 4, 5, 6, 8; (*c*) 9, 12, 14, 15, 17; (*d*) 10, 11, 13, 20, 22, 24; (*e*) 16, 18, 19, 21, 28; (*f*) 23, 25, 27, 29, 31, 32. It should be remembered that the presence of large amounts of SSS tends to suppress the reaction and so in some cases it may be necessary to dilute the material so as to bring down its concentration.

When pneumococci are scanty in a specimen, mouse inoculation may be resorted to for concentrating them. A small portion of the material is inoculated intraperitoneally into a white mouse and the peritoneal fluid is aspirated four to six hours after the inoculation. The pneumococcus multiplies rapidly in the peritoneum and the exudate therefore contains large numbers of them besides a rich content of SSS. The organism is then typed by the capsular reaction.

The agglutination test is carried out either with a pure culture after isolation or with the peritoneal fluid from the mouse inoculated with the pneumococcus-containing material. The latter has to be centrifuged and the deposit resuspended in normal salt solution; this forms a suitable suspension of the organism for agglutination test. Agglutinating sera against all the types are now available. The macroscopic or the microscopic method may be adopted.

The precipitation test is done with SSS as the antigen. A watery extract of the sputum containing pneumococci or the cell-free peritoneal exudate of the inoculated mouse may be used (p. 261).

Toxin Production. This organism does not produce any soluble toxin analogous to those of the diphtheria and tetanus bacilli. Under certain conditions it produces a filterable haemolysin which is oxygen sensitive; its function is not known. Others have reported the production of a leucocidin and a purpura-producing substance. But none of these would appear to contribute materially to the virulence of pneumococci. There is no

doubt that the virulence of the microbe is, in some measure at least, dependent on the specific soluble substance and the toxic symptoms in pneumococcal lesions may be the result of its action on susceptible tissues after absorption. It is present in the sputum, urine and the blood of pneumonia patients.

Antipneumococcal serum is prepared from the horse. Suitable animals are inoculated intravenously with killed young cultures of the organism for prolonged periods. The serum is type-specific. It has protective properties on rabbits and mice and has also some curative value. The immune serum is also sometimes known as Felton's serum. The serum can be concentrated and refined. The standardisation of serum is done by the mouse protection test. A unit of serum is the least amount that will protect mice against one million lethal doses of a pneumococcal culture.

Antipneumococcal serum has been recently prepared in rabbits. It is said to be more potent and productive of less reaction. As the antibody molecules in this case are smaller, absorption may also be more rapid. Rabbit serum is also cheaper.

Immunity. The nature of immunity in pneumococcal infections is not well understood. Active immunity against the pneumococcus can be produced in experimental animals, but it is of short duration; it is accompanied by the presence of circulating antibodies, such as precipitins, agglutinins and others. In man also antibodies are developed in response to pneumococcal infections and the immunity produced is trivial and evanescent. There is some evidence to show that these antibodies are in some way concerned in the recovery; in what precise way they are so instrumental is not quite clear. Antisera prepared against the various types of pneumococci vary in therapeutic value; with some types the results are good.

Pathogenicity. *Diplococcus pneumoniae* is pathogenic to man and some of the experimental animals. In man the pneumococcus causes lobar pneumonia, one of the most important acute infectious diseases. Other lesions caused by pneumococci in man are bronchopneumonia, empyema, otitis media, meningitis, conjunctivitis, ulcerative endocarditis, peritonitis, synovitis, etc. A primary meningitis due to *Diplococcus pneumoniae* is not of very rare occurrence; in fact, next to lungs the pneumococcus appears to have the greatest predilection for the meninges.

All the various types are not equally virulent or invasive. The first three are much more virulent and much more invasive than the rest. Of these three, Type III is the most virulent and Type I the least, whereas their invasiveness appears to be in the reverse order, the first being the most invasive and the third the least.

The strains isolated from primary pneumococcal lesions, other than lobar pneumonia, and also those from mild cases of the latter disease belong to the less virulent types, comprising the old miscellaneous Group IV. Infection with these may be exogenous or endogenous. Healthy carriers of these types are common. Sometimes these types are referred to as "carrier" types. But it should be noted that occasionally severe lobar pneumonia may be due to one of these carrier strains and, on the other hand, the virulent strains are, though rarely, seen in healthy carriers. Over fifty per cent. of healthy people show pneumococci in their upper respiratory passages and mouth. The percentage of carriers is higher during the colder months of the year than at other times. A higher percentage is also found among contacts than among non-contacts. The carrier state is not constant but intermittent; individual carriers may carry the infection for varying periods of time. Minor infections from other causes, such as a cold, help to prolong the carrier state. A carrier may harbour more than one type at a time; up to seven have been reported. Carriers of the more virulent or infective types are rare and, when found, are probably only contact carriers. These types are almost always associated with disease. Infection with them is mostly exogenous and is usually followed by disease. A relatively high carrier rate with these strains is also associated with a high incidence of pneumonia.

Different types of pneumococci, and even different strains of the same type, may evince differences in their virulence for different laboratory animals. In general, mice and rabbits are very susceptible to pneumococci and to a less degree guinea-pigs. The organism is very invasive and by whatever route it is given, following local suppuration a septicaemia is soon established; the organism does not show any partiality for lungs as in the case of man. Lobar pneumonia has been produced experimentally in dogs and monkeys by intratracheal inoculation of the organism. Lesions produced in the monkeys bear a close resemblance to those of lobar pneumonia in man.

Lobar Pneumonia. This is an acute infectious disease occurring sporadically and endemically. Rarely it may also occur in localised epidemics. Practically all cases of acute lobar pneumonia are due to *D. pneumoniae*. In about one per cent. of cases Friedlander's bacillus is found alone in the lesions; other organisms that may rarely cause lobar pneumonia are the haemolytic streptococcus and the staphylococcus. Nearly seventy per cent. of cases of lobar pneumonia in adults are due to Types I, II, and III. More than fifty per cent. of cases are caused by the first two types, the so-called epidemic types; the third type accounts for about ten per cent. of cases. After these the most frequent are Types IV, V, VII and VIII. Type XIV is the most predominant type found in pneumonia of children. No doubt, these figures are subject to slight variations in different countries and even in the same country during different seasons.

The theory has been advanced that lobar pneumonia is the reaction of a previously sensitised individual. The experimental evidence, no less than the clinico-pathological considerations, brought forward in support of this contention is at most only suggestive, and what part, if any, bacterial allergy plays in the causation of lobar pneumonia remains yet a moot point.

Lobar pneumonia is a universal disease. It occurs at any age but more commonly in children up to the age of six years and in young adults. It is a very frequent terminal infection in the aged. It is a striking feature that lobar pneumonia occurs predominantly in the young adult. No satisfactory explanation for this has been given. Man is relatively resistant to the pneumococcus and marked impairment of resistance from such non-specific causes as alcoholism, sudden chill, fatigue, etc. is, in all probability, a necessary precursor of successful infection. An inapparent or mild infection of the upper respiratory tract due to some other organism may pave an easy way for the spread of infection from the nasopharynx to the lungs. Transmutation of types has not been proved to occur in nature. There is a well marked seasonal incidence of lobar pneumonia, practically all cases occurring during the colder months of the year when the carrier rate is also the highest.

Infection with the virulent strains are largely exogenous in origin, active cases and possibly infected dust forming the source of infection. Droplet infection undoubtedly plays an important

part in the transmission of the infection. The high incidence of lobar pneumonia due to Types I and II in contrast to the low carrier rate of these types would seem to support the view that infection with these types is largely exogenous. Carriers of these types are uncommon and not so important as sources of infection as carriers of the less virulent types, who are quite common. Carriers may themselves develop the disease when the host conditions become favourable or they may transmit the infection to others.

There is overwhelming evidence to show that pneumococci enter through the respiratory route. The older view was that the organism first invades the blood stream by some route or other and then settles down in the lungs. As the result of their investigations on monkeys, Blake and Cecil were the first to challenge this view. By direct inoculation of pneumococcal cultures into the trachea of these animals, they produced in them a disease closely resembling lobar pneumonia in man. Inoculating the organism directly into the blood stream or subcutaneously failed to produce a similar disease. Their results have been amply confirmed by other workers on a number of animals. The source of infection according to Blake and Cecil is the infected droplet which, after gaining entry by way of the inspired air, passes through the wall of one of the large bronchii near the hilum, settles in the interstitial tissue and sets up local cellulitis which spreads along the perivascular peribronchial lymphatics to the periphery in a fan-like manner. Invasion of the alveoli from without then follows, setting up an inflammatory reaction; in serious cases secondary septicaemia develops. From these and other investigations there can be no reasonable doubt now that the infection reaches the lung through the air passages. But regarding the exact site of the primary focus of infection, whether in the hilar interstitial tissue or elsewhere, we are on less firm grounds. More recent work, however, on dogs by Terrell, Robertson and Coggeshall and on a number of other experimental animals by others sseems to dispose of the theory of interstitial spread and confirm a different view previously put forward by Loeschcke. This view is that the primary focus is not in the interstitial tissue nor in the hilar region but in the alveoli in the extreme periphery and that the inflammatory oedematous fluid, teeming with pneumococci, regurgitates as it were from this site and passes

on from alveolus to alveolus directly through the pores of Kohn and to more distant parts through the smaller air passages, thus involving a large portion of the lung tissue.

Secondary invasion of the blood stream by the organism is of frequent occurrence in pneumonia. In a good proportion of cases the pneumococcus can be isolated from blood even in the initial stages of infection. Septicaemia is commonest in the most invasive Type I infection. When septicaemia occurs in the less invasive Type III cases, the prognosis is grave. There need be no correlation between the severity of the lung condition and the degree of septicaemia. There may be cases with persistent and severe septicaemia, ending in death, which show but relatively slight pulmonary changes. Prognosis in lobar pneumonia is determined not so much by the extent of lung inflammation as the degree and persistence of septicaemia, fatality being considerably higher when there is blood infection.

Lobar pneumonia is marked by intense toxaemia, varying in degree with the virulence of the invading type of pneumococcus. What constitutes this toxin, is not apparent. The pneumococcus does not produce a soluble toxin. There is some evidence to show that the specific soluble substance is the cause of this toxaemia. It can be demonstrated in the blood, sputum and the urine of patients. Type III which forms the greatest amount of SSS also causes the most severe form of toxaemia. The toxaemia is less with Type II which forms less SSS than Type III and least with Type I which forms the least amount of SSS. This is suggestive that the specific soluble substance may be responsible for the production of toxaemia. Type I is the most invasive; it is also the most antigenic and the least fatal of the three. A brisk response on the part of the tissues followed by a sharp crisis is a marked feature of this type. Type II is more fatal and Type III most. The specific soluble substance does not seem to exercise any direct toxic action on phagocytes. Sensitisation of pneumococci by the opsonins and the bacteriotropins is an essential prerequisite for phagocytosis, and it has been suggested that the specific soluble substance interferes with the phagocytic action by neutralising these sensitising antibodies (p. 204). Marked leucocytosis is generally an early tissue response in lobar pneumonia. A poor leucocytic reaction points to an unfavourable prognosis.

Specific antibodies against pneumococci are formed by the tissues and are liberated into the circulating blood. They are elaborated from the early stages of the disease, attaining a high maximum titre by the time of crisis after which the titre is maintained in the same level for some time. The presence of the specific antibodies in the patient's serum can be demonstrated either by the protection test on mice or by the precipitation test employing SSS as the antigen. An intradermal test with dilute SSS is also available for testing the presence of antibodies in the blood—Francis test. An urticarial wheal with a zone of erythema is a positive reaction and indicates the presence of antibodies.

The mortality rate in lobar pneumonia is relatively high. It is influenced greatly by the type of the invading pneumococcus. Generally, the fatality rates are the highest with Types I, II and III. Of these, Type III pneumonia is the most fatal form, causing a death rate of 40–60 per cent. Types II and I show a progressively falling mortality rate, from 40–25 per cent. All the other types put together cause a 15–20 per cent. fatality rate.

Diagnosis. Examination of smears of materials from patients, such as pus, sputum, cerebrospinal fluid, etc., stained by the Gram's method shows the typical violet stained diplococci, the capsule presenting the appearance of an unstained halo round each pair. Staining methods alone may not be enough, particularly with specimens like sputum. Cultures are made on blood agar or in buffered glucose broth if the specimen is blood. The colony characters and the alpha type of haemolysis provide additional diagnostic evidence. But it may be reminded that the viridans streptococcus also develops greenish colonies on blood agar. Further, this streptococcus also sometimes shows a tendency to grow in pairs and the pneumococcus, on the other hand, may show a tendency to grow in chains. For these reasons, the differential diagnosis of the pneumococcus from the viridans streptococcus sometimes presents real difficulty. Two reliable tests for identification are inulin fermentation and bile solubility. The polysaccharide inulin is almost always attacked by the pneumococcus, whereas the very vast majority of viridans strains do not ferment this sugar. More certain than this is the bile solubility test. Bile dissolves the pneumococcus but not the viridans streptococcus. A positive agglutination reaction with the specific serum is conclusive.

Diagnostic inoculation is not necessary in the vast majority of cases. A small portion of the suspected material, *e.g.* pneumonic sputum, is injected subcutaneously or intraperitoneally into a mouse or rabbit. A septicaemia, fatal in twenty-four to forty-eight hours, rapidly develops. The heart blood shows the typical capsulated organism in large numbers. Apart from the point of view of diagnosis, inoculation of the mouse also serves to concentrate the organism, when it is scanty in a specimen, for serologicals tests (p. 396). The identification of the type of the infecting pneumococcus is an essential preliminary to serum therapy. It is discussed in a previous section.

Prophylaxis. Some observers regard that active immunisation with vaccines confers a certain amount of protection against pneumonia. In any case the multiplicity of types is a great handicap, limiting considerably the usefulness of vaccines. The specific soluble substance has been employed for active immunisation, but the results are not encouraging.

Treatment. Specific serum therapy has some value in the treatment of pneumonia. It is handicapped by the multiplicity of serological types. It is most successful in Type I cases wherein the improvement rate is claimed to be about sixty per cent. The success in Type II cases is very much less than in Type I and serum is of little value in Type III infection. Good results have also been claimed in Types V, VII and VIII infections. Early typing of the infecting pneumococcus is of supreme importance. The serum must be given as early as possible in the disease. If serum administration is decided upon, a polyvalent antiserum, prepared against the more prevalent epidemic types, must be given immediately and the treatment should then be continued, after typing, with the appropriate monovalent serum. The dose should be adequate and the route intravenous. The initial dose may be 10,000–20,000 units to be repeated every 8–12 hours until definite improvement follows. Up to a total of 100,000 units may be given in any single case. The serum is very costly. Sensitivity to normal horse serum must be previously tested for and, if present, overcome by desensitisation. Wheal or urticarial test: 0·1 c.c. of horse serum injected intracutaneously produces an inflammatory area at the site within forty-eight hours. Conjunctival test: a drop of a 1 : 10 dilution of horse serum instilled into the conjunctival sac produces lacrimation, redness and itching in ten minutes.

Heat-killed vaccines have been employed for therapeutic purposes. In practice they are of doubtful utility.

Chemotherapy. In pneumococcal infections also chemotherapy has largely relegated serum therapy to a secondary place. Sulphonamide compounds are very effective in the treatment of pneumococcal infections. Penicillin has further improved the outlook in these conditions. A combination of chemotherapy and serum treatment may yield better results than either alone. Drug-fast strains of pneumococci are sometimes developed as a result of sulphonamide therapy.

CHAPTER XIX

NEISSERIA

The genus *Neisseria* contains Gram-negative cocci, usually occurring in pairs with the adjacent sides flattened and often growing poorly on ordinary media, but growing well on serum media. They are strict parasites and live on mucous membranes. Some of them are pathogenic. Many are found as non-pathogenic inhabitants particularly of the mouth and upper respiratory tract of man. *Neisseria gonorrhoeae* and *Neisseria intracellularis* are the best known species.

Neisseria gonorrhoeae was the first member of the genus to be described. Neisser (1879) observed paired cocci in the pus from cases of gonorrhoea. Due to its constant association with this condition and due to the absence of any other organism to account for it, he arrived at the conclusion that it was the cause of gonorrhoea. The organism was isolated in pure culture by Bumm (1885). *Neisseria intracellularis* was the next species discovered. Marchiafava and Celli (1884) had described the occurrence of Gram-negative diplococci in the spinal fluid of meningitis cases. But it was Weichselbaum (1887) who first isolated the organism from the spinal fluid of patients suffering from cerebrospinal meningitis and described it in detail. The causal relationship of *N. intracellularis* to epidemic meningitis was subsequently confirmed by other workers. Since then, several Gram-negative cocci have been described from the healthy as well as diseased nasopharynx of man: *Neisseria crassus*, *Neisseria catarrhalis*, *Neisseria sicca*, *Neisseria flava* and others. *Neisseria flavescens* is another organism recently isolated from the spinal fluid of some cases of cerebrospinal meningitis in America.

Classification. With the exception of two species, *N. intracellularis* and *N. gonorrhoeae*, the Gram-negative cocci are very little studied and there is no reliable bases for the differentiation of species. Fermentation reactions are now relied upon for this purpose. While the gonococcus ferments only glucose and the

meningococcus both glucose and maltose, with no gas production, *N. catarrhalis* attacks none of the sugars (p. 418). Pigment production is also helpful in defining a few species.

Neisseria Gonorrhoeae. The gonococcus, the causative organism of gonorrhoea, was first described by Neisser in 1879. It is a strict parasite of man found only in the specific lesions which it causes, wherefrom it may frequently invade the blood stream.

Morphology and Staining. Gonococci are oval or spherical in shape, 0·6–1·0 μ in diameter and arranged typically in pairs. The opposed surfaces are flattened and usually slightly concave and the long axis of the pair is at right angles to the axis connecting the two cocci, giving a reniform or coffee bean appearance to the organism. The occurrence of single cells and involution forms is very common in cultures, the latter due to autolysis. Such aberrant forms are also encountered in chronic leisons. Gonococci are generally intracellular, clustered up in enormous numbers in pus cells; as many as a hundred may be seen in a single cell. They are non-capsulated and not motile. They take the ordinary stains without difficulty and are Gram-negative and non-acid-fast. Staining is uneven in older cultures and in materials from chronic cases.

Growth Requirements. The gonococcus is essentially an aerobe, little or no growth occurring under strict anaerobic conditions. A 10 per cent. CO_2 tension seems to improve growth. The optimum temperature is 37° C.; no growth occurs below 30° or above 39° C. The optimum pH is 7·5.

The cultivation of the gonococcus is not easy. It does not grow on ordinary media. The addition of animal proteins, such as blood, ascitic or hydrocele fluid, to the medium is essential for growth. A 10 per cent. heated blood agar or ascitic agar made with infusion broth is a satisfactory medium. Drying is inimical to its life and the presence of plenty of moisture has to be ensured in the medium for growth. Outside the tissues, the gonococcus remains viable only for a short time. Therefore, immediate insemination of the infectious material into the medium is essential for success. Growth may be slow.

Cultural Characters. The colonies on solid media are small, about the size of a pin head, round, translucent, finely granular with lobate margin and greyish white with a pearly opalescence by transmitted light. In serum broth growth is very poor, giving

rise to little or no turbidity and a slight granular deposit. Autolytic changes set in rapidly and distorted forms are by no means rare in fluid cultures.

Biochemical Activity. This organism exhibits considerably restricted fermentative capacity. It produces acid without gas from glucose but not from other common sugars. Indole is not formed and no change is produced in litmus milk. Catalase production is marked; the production of indophenol oxidase is turned to practical use for the identification of gonococcal colonies. The growth on heated blood agar, after two days incubation, is flooded with a one per cent. solution of tetramethyl-*p*-phenylenediamine hydrochloride which is drained off immediately. Gonococcal colonies, in fact colonies of all bacteria that form indophenol oxidase, develop a rich purple colour—the so-called *oxidase reaction*. The addition of the reagent does not destroy the organism immediately and subcultures may be successful if done within half an hour after pouring the solution.

Resistance. The gonococcus is a delicate microbe readily destroyed by adverse agencies, like heat, light and chemicals. Moist heat at 55° C. destroys it in less than five minutes. Drying under ordinary conditions is rapidly lethal to its life. Outside the tissues the organism perishes soon, in about an hour or two. However, viability may be retained up to three days or more on linen soiled with gonorrhoeal discharge. Dilute antiseptics destroy gonococci very rapidly; a one per cent. phenol, for instance, kills them in one to three minutes. The organism is likewise highly sensitive to certain dyes and silver salts. It is very susceptible to its own ferment and so autolytic process is a very conspicuous feature. For this reason, the preservation of stock culture is not easy. When cultivated on suitable media provided with plenty of moisture and kept at 37° C., it may survive up to a week. Freeze-drying is another satisfactory method of preserving cultures.

Variation. Two different types of colonies have been observed: a large, irregular, flattened, transluscent colony on which papillae develop on continued cultivation—Type I, and a smaller round, slightly raised, low convex, opaque colony, forming no papillae—Type II. Type I colonies are formed generally from pus from acute gonorrhoea and Type II in old laboratory cultures and from materials from chronic cases. There are probably inter-

mediate types and the papillae formation may be the initial step towards transformation to Type II. These colony types seem to agree with the serological types.

Antigenic Structure. The gonococcus shows a complex antigenic structure. Recent work on antigenic analysis of the organism indicates the presence of polysaccharide and nucleoprotein fractions which are common to other members of the *Neisseria* group. The occurrence of type-specific carbohydrates has also been reported. Gonococci do not form a serologically homogeneous group. On the basis of agglutination and agglutinin absorption tests, two main serological types have been described—Type I and Type II. Strains isolated from acute lesions correspond to Type I and those from chronic lesions fall under Type II. These immunological types are correlated with the cultural types mentioned above.

Toxin Production. *N. gonorrhoeae* does not form any soluble toxin. Endotoxin is, therefore, what is responsible for pathogenesis. Due to rapid autolysis fluid cultures may contain varying amounts of the toxic material. The endotoxin is fairly toxic to experimental animals, like mice, guinea-pigs and rabbits; large doses, intraperitoneally injected, kill the test animal rapidly. The organism does not seem to multiply in the animal body.

Pathogenesis. It is a strict parasite of man and causes the primary urethritis called gonorrhoea. From the primary seat the infection may spread rapidly along contiguous mucous surfaces and give rise to secondary lesions, such as prostatitis, epididymitis, vesiculitis, cervicitis, inflammation of the Bartholin's glands, salpingitis, pyosalpinx, peritonitis and other inflammatory conditions. Proctitis is also a common complication, particularly in women and female children. Gonorrhoeal ophthalmia is relatively rare in adults but serious. Some people regard that the adult conjunctiva is less susceptible than the infant's. From the lesions gonococci may invade the blood stream and cause septicaemia with localisation in the joints, endocardium or meninges. Gonococcal arthritis is a relatively frequent complication. Endocarditis is much less common but more serious. In the newborn the gonococcus causes the condition called ophthalmia neonatorum, the infection occurring from the maternal parts during the passage of the foetus through the birth canal. A persistent vulvovaginitis in female children is often gonorrhoeal in origin. A

primary gonococcal vaginitis does not occur in the adult female, as the squamous epithelium lining her vagina is relatively insusceptible to invasion by this organism. The vaginal epithelium of children is columnar in type and this is very vulnerable. However, it may be remembered that not all cases of ophthalmia neonatorum and vulvo-vaginitis of children are due to gonococci.

Gonorrhoea is an acute infectious disease primarily affecting the genito-urinary tract. It is widely prevalent among all classes of people, regardless of social status. Except in the case of vulvo-vaginitis in children, direct contact, usually sexual, is the mode of spread. The spread in vulvo-vaginitis is commonly by the indirect method through infected towels, bed clothes and the like. A tendency to chronicity is a marked feature of gonorrhoea and, once infected, an individual may remain infective for many years. Even after apparently complete cure the organism may persist in the genito-urinary secretions for a long time, although bacteriological examination may prove negative, and propagate the infection.

Immunity. Little or no immunity is developed as the result of an attack of gonorrhoea and second and third attacks may occur even during the pendency of the first. Antibodies, like agglutinins, precipitins and complement-fixing antibodies, are, however, found in the patient's blood. A marked skin reaction to killed gonococcal suspensions has also been noticed. The role of phagocytosis in gonococcal infections is not well understood.

Diagnosis. The diagnosis of acute gonorrhoea is fairly easy. The organism is present in large numbers in the discharge at an early stage, but later its number is reduced and secondary invaders, like the pyogenic cocci and diphtheroids, make their appearance. Urethral discharge from males and urethral and cervical discharges and materials from the para-urethral or Skein's glands and Bartholin's glands from females should be examined. Vaginal discharge from the infants and children should be examined in suspected cases of vulvo-vaginitis. The purulent discharge from the eye in cases of ophthalmia neonatorum contains the organism usually in large numbers. Examination of a smear, made from the material, after staining by the Gram's method, shows the characteristic Gram-negative bean-shaped diplococci, mostly occupying an intracellular position. Often the pus cells are

crammed with gonococci. Secondary organisms are rare. If the smear examination is positive, a provisional diagnosis of gonorrhoea is justified in most cases. Morphologically identical cocci, it should be kept in mind, may be present in the vagina or the conjunctiva. When there is purulent discharge and a smear of it does not show Gram-negative diplococci, it is fairly certain that the condition is not gonorrhoeal in origin. However, when smears are doubtful or negative, culturing is to be proceeded with. The isolated cultures are then identified by fermentation tests (p. 418, Table X).

In chronic cases the diagnosis is very difficult. The organisms may be scanty and fail to show the paired arrangement, characteristic shape and the intracellular position. In metastatic lesions, like arthritis, the exudates are often sterile. For these reasons, direct examination of smear is inconclusive and cultivation of the organism by suitable cultural methods is essential to establish a diagnosis (p. 300). Even the latter procedure is very difficult, especially in the female. In the male the "morning drop" of secretion from the urethra and the prostatic fluid, obtained after massage, and in the female materials collected from the situations mentioned above are to be used for culture. Cervical discharge should be taken through a vaginal speculum as, otherwise, contamination from the vagina will render isolation unusually difficult. Endeavour should be made to inseminate the material directly from the patient into the medium; if this is not possible, inoculation should be done without any delay after taking it, incubating the culture medium immediately after. The oxidase reaction described above is helpful in identifying the colonies of gonococcus from a mixed growth. It is followed up by the examination of smears from the purple colonies by the Gram's method. By this procedure isolation also is rendered easier. Subcultures from the identified colonies are successful if made immediately after pouring the solution. The cultures are then put through sugar reactions. More recently a microscopic test, based on the solubility of gonococci in N/10 NaOH, has been described for the further differentiation of gonococcal colonies from colonies of other *Neisseria*.

Serological Methods. The complement-fixation test is of value in the diagnosis of chronic cases. Precipitation and skin tests give irregular results and are but seldom employed. It is

claimed that in the first week of the disease 27 per cent. of cases give a positive complement-fixation reaction, rising to hundred per cent. by the end of the fifth week. An extract of the organism, prepared by treating it with KOH followed by neutralisation, forms the antigen. A number of freshly isolated strains should be included for extraction. The general technique followed is the same as that for the Wassermann test. When interpreting the results, it should be remembered that a positive reaction is also caused as the result of vaccine treatment. But it is evanescent. Repeated negative results in a treated case, in which the test had been previously positive, indicate cure.

Prophylaxis. The prophylaxis of gonorrhoea consists in the avoidance of infection. Moral and personal cleanliness is the most important factor in prevention. Early diagnosis and efficient treatment also help in preventing spread. Specific immunisation is of no use in the prevention of infection. The use of antiseptics after exposure may have some value; a solution of argyrol or protargol is instilled into the urethra. The routine method of instilling solutions of silver compounds into the eye immediately after birth has been very successful in reducing the incidence of ophthalmia neonatorum.

Treatment. Vaccine therapy has some adherents. It is not universally regarded as effective. Many consider it as useless if not harmful, while others claim that it is a very valuable adjunct, particularly in the treatment of complications and in chronic cases. It seems to give a good account when used as a provocative to flare up a chronic condition. Antigonococcal sera have not proved successful in the treatment of gonorrhoea.

Chemotherapy has entirely altered the problem of the treatment of gonococcal infections. Sulphonamides, particularly sulphathiazole and sulphadiazine, have proved efficient remedies in gonorrhoea. However, their use is not uniformly successful and sulphonamide resistant cases are not infrequent. Penicillin is a better remedy than sulpha compounds and its success in the treatment of sulphonamide resistant cases is particularly fortunate. Pyrotherapy and other drastic and troublesome measures have been rendered unnecessary by the introduction of the sulpha compounds and penicillin.

Neisseria intracellularis. The meningococcus has been called by several names by different authorities. Weichselbaum (1877),

who first isolated it from the meningeal exudate derived from cases of cerebrospinal meningitis, called it *Diplococcus intracellularis meningitidis*. Some of the other names subsequently used are *Micrococcus intracellularis meningitidis*, *Micrococcus meningitidis* and *Neisseria meningitidis*.

Habitat. It is an obligatory parasite of man and its natural habitat is the nasopharynx. It is found in the cerebrospinal fluid, blood, nasopharynx and rarely other situations in patients suffering from cerebrospinal meningitis.

Morphology and Staining. Meningococci are spherical cells, 0·6–0·8 micron in diameter, occurring singly, in pairs with flattened opposing surfaces or occasionally in tetrads or groups. The meningococcus resembles the gonococcus closely. In the meningeal exudates the paired arrangement and intracellular position are both characteristic. But even in the same specimen variability in size may be a marked feature. In cultures too the typical arrangement is often lost and great variations in shape and size are encountered. Meningococci are devoid of capsule and flagella. They are readily stained by simple stains and are Gram-negative and not acid-fast. Irregular staining, particularly of old cultures, may be noticeable.

Growth Requirements. The organism is an aerobe and does not grow under strict anaerobic conditions. The presence of 10 per cent. CO_2 is said to enhance growth. The optimum temperature is 37° C. with a range of 22°–40° C. Below 30° C. growth is very poor. The optimum pH is 7·4–7·6.

The meningococcus is less difficult to grow than the gonococcus. Enrichment of the simple media with natural proteins is necessary for growth. Serum agar and blood agar media are satisfactory. The addition of glucose to these improves the growth further. Chocolate agar or blood agar with infusion base seems to give the best results.

Cultural Characters. On serum agar the colonies after twenty-four hours are small, just more than 1 mm. in diameter, slightly convex, transparent and glistening with a bluish sheen. Older colonies may be opaque and granular in the centre, slightly yellowish in colour and having a crenated periphery. The colonies are butyrous in consistence and easily emulsifiable. Old cultures are less exacting in their food requirements and may grow on ordinary agar or glucose agar. Growth in serum broth causes

moderate turbidity and slight granular deposit. Frequent transplantation is necessary to keep the organism alive. On Dorset's egg medium it grows poorly but remains viable for a number of days.

Biochemistry. As in the case of *N. gonorrhoeae*, the fermentative capacity of the meningococcus is restricted. It attacks both glucose and maltose, stopping short with the production of acid. The action on maltose differentiates it from the gonococcus. There is no change in litmus milk and indole is not produced.

A weak haemolysin is produced and some strains show a slight yellow pigmentation of colonies. Catalase production is positive and autolysis is a marked feature; the organism dies out quickly in culture. This is less marked on egg medium.

Resistance. The meningococcus is a delicate organism and is highly susceptible to inimical agencies. Desiccation is particularly destructive. When dried under ordinary atmospheric conditions, the organism dies out in two to three hours. It is killed by moist heat at 55° C. in less than five minutes and by one per cent. phenol in one minute. Sealed cultures made in suitable media and kept at 37° C. may remain viable for four or five weeks.

Antigenic Structure. From the available evidence it appears that certain antigenic constituents are shared not only by *N. gonorrhoeae* and *N. intracellularis* but also by other members of the group. The antigenic structure of the meningococcus is complex and the species does not constitute a serologically homogeneous group. Gordon and Murray classified the organism into four types on the basis of agglutination test. Types II and IV are closely related and exhibit marked cross agglutination. Other workers consider that all the known strains can be brought under two groups, Groups I and II, there being little serological distinction between Gordon's Types I and III and Types II and IV. In general, Type I seems to be associated with the epidemic form of the disease and Type II predominates in the interepidemic periods. Recently, a new type with resemblance to Type II, and therefore called Type II *a*, has been described.

Toxin Production. No soluble toxin is known in the case of *N. intracellularis*. Endotoxin seems to be its weapon of offence. Presumably, it is responsible for the profound toxaemia seen in meningococcal meningitis. Due to autolysis powerful toxic products are liberated in cultures. Recently, claims have

been put forward demonstrating the presence of an extracellular toxin in the cultures of meningococci. But they have not been confirmed.

Pathogenicity. Meningococcal infections are confined to man. Experimentally, when fairly large doses are injected intraperitoneally into mice, guinea-pigs or rabbits, the animals die of toxaemia in one to four days. The organism does not seem to multiply in the animal body.

The meningococcus has a selective affinity for the meninges and causes sporadic and epidemic cerebrospinal meningitis. The posterior basic meningitis of children, which occurs mostly in sporadic form, has also the same aetiology. It is being increasingly recognised that the meningococcus may also cause subacute and chronic septicaemia without meningeal involvement unless as a complication.

Besides *Neisseria intracellularis*, a variety of micro-organisms may cause inflammation of the meningeal investments of the cerebrospinal axis. Mostly, this type of infection is secondary to some primary foci situated elsewhere in the body. The common organisms may be the pneumococcus, streptococcus, tubercle bacillus, staphylococcus, influenza bacillus, typhoid, colon and plague bacilli. Of all these, meningococcal meningitis is the only type which occurs in epidemic form.

Meningococcal meningitis is endemic in many of the large towns and cities and may take an epidemic turn. Sporadic cases occur at intervals in these places, serving as a connecting link between epidemics. Epidemics of meningococcal meningitis are peculiar in that they consist of a series of small recurrent outbreaks spread over a longer time instead of a single violent wave sweeping through within a short period as in other epidemics. Again, in an epidemic area the cases are widely scattered about also in point of place rather than clustered together like other epidemics.

The infection is air-borne by the droplet method. Direct contact may also transport it. The organism enters through the upper respiratory tract and reaches the nasopharynx where it may or may not produce a mild rhinopharyngitis. In either case the organism settles down there and a carrier condition is established. Carriers may be temporary or permanent. The organisms are discharged in the nasopharyngeal secretions; it may

not be continuous but intermittent, sometimes with long intervals.

Carriers are mainly concerned in the transmission of infection. Epidemics of meningococcal meningitis are sometimes referred to as "carrier epidemics". There is indeed a pre-epidemic increase in the carrier rate which may even reach 50 per cent. or more. Some workers regard that it is only after such a saturation point is reached do epidimics start. Among the military populations an enormous pre-epidemic increase of the carrier rate, from 2–4 per cent. of the normal times to as high as 80–90 per cent. during the height of epidemics, has been reported by many investigators. The possible sources of infection are the healthy carrier, the convalescent carrier and the active case. Of these, the healthy carrier appears to be the most frequent cause of transmission. Active cases are not important transmitters of the infection. Persons who come in contact with active cases may also acquire the infection and become temporary or permanent carriers but seldom develop the disease. Another source of spread is the clinically unrecognised, mild and abortive cases; such cases do undoubtedly occur.

Overcrowding, such as occurs in barracks, hostels, schools, theatres and similar institutions, favours rapid diffusion of the infection. Due to the same cause the infection is wide spread in large cities and towns. Conditions obtained during cold weather, such as closer association of people and reduced ventilation, are also conducive for the rapid spread of the infection. Also, during the cold weather many people suffer from a mild catarrhal condition of the upper respiratory tract, which, by reducing the local resistance of the nasopharynx, may facilitate an easy colonisation by meningococci. All these factors contribute to the establishment of a high carrier rate and when conditions become favourable epidemics break out. Some observers consider that epidemics break out only when the carrier rate goes beyond 20 per cent. But the existence of any such relationship between the carrier rate and the onset of epidemics has not been established.

It is only a small proportion of the carriers that develop the disease. The susceptibility of the meninges is apparently low. Hence, there is always a preponderance of carriers over cases. The virulence of the meningococcus and the impaired resistance

of the host are probably the main factors determining the development of the disease. Different strains of meningococci are found to vary widely in their virulence. In general, Group I strains are more virulent than Group II strains; the former are responsible for most epidemics, while the latter predominate during the inter-epidemic periods. Further, it may be that during the pre-epidemic period there is a stepping up of the invasive property of the organism, promoting its spread from the nasopharynx to the meninges and the tissues. In the host such conditions as over-exertion and fatigue, lowering of resistance from any other cause and unhygienic surroundings may also determine the spread of the organism to the meninges. The maximum prevalence of the disease is during winter and spring, coinciding with the peak period of carriers. It is more common in cities and towns than in the rural areas. It is also commoner among children and adolescents. The military populations are particularly prone to develop the disease. Sex and race have no particular influence. However, the incidence is greater in men than in women; this may be an expression of greater opportunities for contracting the infection rather than any difference in susceptibility.

How exactly the organism reaches the meninges from the nasopharynx, is not clear. Two views have been expressed. One is the direct transmission of the germ from the nasopharynx to the meninges, either by direct tissue extension or by the perineural lymphatics of the olfactory nerve filaments transporting it across the perforated plate of the ethmoid bone. According to this view, the invasion of blood, which undoubtedly occurs, is a secondary phenomenon. The other view is that the blood is invaded directly from the nasopharynx and an initial septicaemia occurs followed by localisation in the meninges. The involvement of the meninges, if this view is the correct one, is a secondary event. Haematogenous route is supported by the fact that blood culture is often positive in the early stages of the disease before any meningeal symptoms appear; as also by the occurrence of meningococcal septicaemia without meningeal involvement.

On reaching the meninges, the organism multiplies and apparently sets free a powerful endotoxin which causes an acute inflammation of this structure followed by purulent meningitis. Meningococci are invariably present in the cerebrospinal fluid

which may vary in appearance from a slight turbidity to the consistency of actual pus. The organisms may be few or in large numbers, both free and within the leucocytes. When blood infection occurs, haemorrhages into the skin is a common feature and petichiae appear. It is this that has given the disease one of its common names—spotted fever. Meningococci may be found in these rashes.

Cerebrospinal meningitis is a serious disease causing low morbidity but a high case mortality which is about 70 per cent.; it ranges between 35 and 90 per cent. in individual epidemics. The introduction of serum treatment has reduced the mortality rate by fifty per cent. and sulphonamide therapy has reduced it still further to about 10 per cent. The highest mortality is among infants and the lowest in children between 5 and 10 years of age.

Immunity. Antibacterial antibodies are produced during the course of the disease, but they are too irregular to be of diagnostic value; they also disappear rapidly from the blood. How long the immunity lasts after recovery from the disease is not known. Second attacks rarely occur.

Diagnosis. The spinal fluid, blood and nasopharyngeal swabs are the materials available for examination. Of these, the first is the most important. The spinal fluid is usually turbid and loaded with pus cells. Direct examination of films made from it and stained by the Gram's method shows the Gram-negative diplococci, mostly intracellular but also outside the pus cells. On the slightest suspicion of a case, drainage of the cerebrospinal cavity must be done. It is also a therapeutic measure. The detection of a *Neisseria* organism in the cerebrospinal fluid is practically certain of the diagnosis, especially during an epidemic. The gonococcus may cause meningitis, but it is exceedingly rare. In the very early stages of the disease and also at a later stage meningococci may be very scanty in the cerebrospinal fluid which may be quite clear.

Cultivation of the organism is the next procedure. For this purpose a specimen of the fluid should be transferred to the laboratory without any delay and direct inoculation made into suitable media. Should the fluid happen to be clear, a centrifuged deposit is to be used for culture; or the whole fluid should be incubated for 24 hours before making cultures from it. This enables the organism to grow in the cerebrospinal fluid. The isolated orga-

nism can be identified by its staining reactions, colonial appearances and by its action on glucose and maltose. The agglutination test with the type-specific sera may be employed for typing, when necessary. Isolation of the organism by blood culture also may be attempted in the early stages or when meningeal symptoms are absent. In the early stages of the disease and in the case of carriers, cultures may be made with materials taken from the posterior nares and the posterior wall of the nasopharynx. During the act of taking, the swab should not come in contact with any other surface. West's swab is specially designed for this purpose. Cultures should be made immediately on serum agar or infusion blood agar and incubated without delay. The meningococcus also is oxidase-positive and the oxidase test may be used for identifying its colonies in the nasopharyngeal cultures. The identification of the organism isolated from the nasopharynx should be finally confirmed by agglutination with a polyvalent or monovalent specific serum.

TABLE X

Fermentation Reactions of the Gram-Negative Diplococci

Neisseria	Glucose	Maltose	Saccharose	Levulose	Mannite	Lactose
N. intracellularis ..	A	A	—	—	—	—
N. gonorrhoeae ..	A	—	—	—	—	—
N. catarrhalis ..	—	—	—	—	—	—
N. sicca	A	A	A	—	—	—
N. perflava	A	A	A	A	A	—
N. flava	A	A	—	A	—	—
N. subflava	A	A	—	—	—	—
N. flavescens ..	—	—	—	—	—	—
D. mucosus	A	—	—	—	—	—
D. crassus	A	A	A	A	—	A

The precipitation test is a rapid method and when positive reliable. A few drops of the supernatant fluid from a centrifuged specimen is layered gently on a small column of the specific serum in a Dreyer's tube. In positive cases a white opaque ring is

formed at the surface of contact in a few minutes. If immediate reaction is not obtained, the tube is incubated in a water bath at 37° C. for one to two hours.

Prophylaxis. Specific immunisation is of no value. Measures preventing overcrowding and physical overexertion may reduce the incidence. It is important to maintain a healthy nasopharynx. The early detection and treatment of carriers and cases are also essential in prevention.

Treatment. Spinal drainage and serum therapy were the mainstay of treatment till the introduction of the sulpha compounds. Serum is of definite value. Since its introduction by Flexner, the fatality rate has been reduced from 70 to 30 per cent. It is an antibacterial serum and is prepared by the active immunisation of horses first with killed and later with living meningococci. Uniform and accurate method of standardisation is not available due to the absence of any susceptible animal. Standardisation on the basis of mouse protection has been suggested. Both type-specific and polyvalent sera are available and where typing is not possible or delayed the latter should be used. The dose is 30–60 c.c. in adults and proportionately less in children. It should be given once or twice a day for four to five days, depending upon the seriousness of the case. It is injected into the cerebrospinal cavity either by the lumbar or the cisternal route. The serum should be warmed to the body temperature and the amount administered should be slightly less than the amount of cerebrospinal fluid drained. It should be given immediately the diagnosis is made or as early as possible. The intrathecal administration may be reinforced through the intravenous route. Recently, antimeningococcal serum prepared in the rabbit has been said to be superior to that prepared in the horse.

Chemotherapy has considerably improved the outlook in meningococcal meningitis. The sulphonamide compounds are highly effective in the treatment of this disease. Many workers advocate a combination of chemotherapy and serum therapy. Penicillin is lethal to meningococci. Administered intravenously or intramuscularly in sufficient doses, penicillin seems to reach the cerebrospinal fluid in adequate concentration, rendering intrathecal administration of it unnecessary; but spinal therapy may be safer.

Other Neisseria Organisms. A heterogeneous group of Gram-negative cocci exhibiting slight differences in their colonial appear-

ances, biochemical reactions and metabolic activities are found as commensals in the upper respiratory tract. They have not been much investigated nor classified satisfactorily. Most of them exhibit greater biochemical activities than the pathogenic *Neisseria*. In association with other organisms they may be frequently encountered in many of the inflammatory lesions of the mucous membrane. Two species designated *Diplococcus mucosus* and *Diplococcus crassus* are described under *Neisseria* by some authorities. The former is probably a rough variant of some *Neisseria*. The inclusion of the latter in this genus is open to question.

Neisseria catarrhalis. This is a Gram-negative coccus arranged in singles, pairs, tetrads or small clumps. Its natural habitat is the upper respiratory tract, and in common cold and many other respiratory infections it is found as a secondary invader in association with the causative agents.

In morphology and staining reactions it resembles the meningococcus. As a rule, it is somewhat smaller than the latter. But in tissues *Neisseria catarrhalis* may sometimes appear slightly larger than the meningococcus and the latter slightly larger than the gonococcus.

It grows readily on all media, but a few strains require enrichment with blood or serum for the first few cultures. The colonies are larger and more opaque than those of the meningococcus. They tend to undergo very rapid variation. In broth growth occurs as a scaly or granular deposit. The organism grows rapidly at room temperature. Biochemically it is inert.

Neisseria catarrhalis is either non-pathogenic or of low pathogenicity. It is often regarded as causing catarrhal inflammation of the respiratory tract. Some strains are pathogenic, like meningococci, for white mice.

Neisseria Sicca. It is a small Gram-negative coccus found on the mucous membrane of the respiratory tract. Its colonies on solid media are markedly dry, tough, very firmly adherent to the medium and almost impossible to emulsify. Hence designated *N. sicca*. It ferments glucose, saccharose, levulose and maltose. Some regard it as merely a rough variant of some other type of nasopharyngeal *Neisseria*.

The Neisseria Flava Group. *Neisseria flava*, *Neisseria perflava*, and *Neisseria subflava* are Gram-negative diplococci also found

on the mucous membrane of the upper respiratory tract in man, possessing chromogenic activity. In morphology they resemble *N. catarrhalis*. They produce greenish yellow colonies on solid media. Their biochemical reactions are given in the table on page 418.

Neisseria Flavescens. This is a chromogenic member. It resembles the meningococcus in morphology but differs from the latter in its production of a golden yellow pigment and in its complete inactivity towards carbohydrates. Serologically it forms a homogeneous group different from the meningococcus; antimeningococcal serum does not agglutinate it. It has been reported from America as the causative organism in some epidemics of cerebrospinal meningitis.

Diplococcus Mucosus. This includes capsulated Gram-negative diplococci growing in mucoid colonies on solid media. It may be a variant of some type of *Neisseria*.

Diplococcus Crassus. This organism grows in minute colonies somewhat resembling those of streptococci. The staining by the Gram's method is irregular, some elements staining Gram-positive others Gram-negative. Its attack of lactose differentiates it from the *Neisseria* group of organisms. It does not seem justifiable to include this species in the genus *Neisseria*.

Veillonella

This genus includes minute Gram-negative cocci, about 0·3 micron in diameter and occurring in masses. The cells are undifferentiated and united by an interstitial substance of an ectoplasmic nature. They are anaerobic and grow best at 37° C. They occur as commensals in the natural cavities of man and animals, particularly the mouth and alimentary tract. It is very doubtful whether they have any pathogenic role, though they may be isolated from the diseased appendix, pyorrhoea alveolaris and pulmonary and other lesions.

CHAPTER XX

BACTERIUM
THE COLON–TYPHOID–DYSENTERY GROUP

Bacterium is a very large genus and contains the coli, enteric, food poisoning and the dysentery groups of organisms besides a host of related species. They are typically intestinal parasites of man and animal but are also found widely distributed in nature, leading a commensal or saprophytic existence. As in the case of the generic name *Bacillus*, the term " Bacterium " is open to objection as a generic title owing to the possibility of its dual application. However, for want of a better and agreed nomenclature, it is necessary to retain it pending future revision. Much chaos exists at present in the nomenclature of this genus, its subgroups and species owing to the indiscriminate use of terms, like *Eberthella*, *Escherichia* and *Klebsella*, that have not received general agreement. The introduction and use of such terms should, therefore, be avoided in order to prevent further confusion.

The members comprising this genus are Gram-negative, non-sporing, rather stout rods with rounded ends, often motile with peritrichate flagella: aerobic and facultatively anaerobic: biochemically very active, producing acid and frequently gas in carbohydrates: not usually liquefying gelatin or coagulated serum.

A satisfactory classification of the several groups of organisms, provisionally included in this genus, has not been arrived at. Several schemes have been proposed from time to time, but none has received universal acceptance. A provisional primary classification, and one that is very convenient and widely employed, is based on the ability of the constituent species to ferment lactose. Thus, there are the lactose-fermenting group, members of which are usually non-pathogenic, and the non-lactose-fermenting group, containing most of the intestinal pathogens. It should, however, be noted that the correlation between the lactose fermentation and pathogenicity is none too rigid, the lactose-fermenters sometimes showing pathogenicity and conversely some

of the avowed pathogens, like certain of the dysentery bacilli, being slow lactose-fermenters. A few species are uncertain in their action on lactose. Further classification is made on the basis of their biochemical activities and clinical manifestations. There is no serological relationship between these different groups.

TABLE XI

A Tentative Classification of the Intestinal Bacteria

1. Lactose-fermenters:

Coli group	*Bact. coli commune* *Bact. coli communior* *Bact. neopolitanum* *Bact. coscoroba* *Bact. acidi-lactici*
Aerogenes group ..	*Bact. aerogenes* *Bact. cloacae*
Friedlander group ..	*Bact. friedlanderi* *Bact. rhinoscleromatis* *Bact. ozoenae*

2. Lactose fermentation variable:

Paracolon group (*Bact. asiaticum*)

3. Non-lactose-fermenters:

A. Salmonella group:

Enteric	*Bact. typhosum* *Bact. paratyphosum A* *Bact. paratyphosum B* *Bact. paratyphosum C*
Food poisoning ..	*Bact. typhi-murium* (Aertrycke) *Bact. cholera-suis* (American Suipestifer) *Bact. enteritidis* (Gaertner)

The paratyphoid group of bacilli may also cause food poisoning.

B. Dysentery group:

Bact. shigae
Bact. ambiguum (Schmitz)
Bact. flexneri
Bact. sonnei
Bact. newcastle
Bact. alkalescens
Bact. dispar

The Lactose-Fermenting Bacteria

The Coli Group

The colon group of bacilli are normal inhabitants of the intestinal canal, especially the large intestine, of man and animals. There are several species but slightly varying from one another. In 1886 Escherich isolated a colon bacillus from the faeces of healthy infants and named it *Bact. coli commune*. Since then, several other closely related species have been found to occur in the intestines. Their constant presence in the faeces and their hardy nature will readily explain their wide distribution in nature, in soil, sewage and water.

Morphology and Staining. The colon bacilli exhibit considerable variation in their morphology. They are straight rods with rounded ends, 2·0–4·0 by 0·5 μ, but long filamentous and short coccobacillary forms may also occur. They are occasionally found in pairs or short chains. Many species are actively motile with peritrichate flagella, while others are aflagellate. A few species are capsulated. Spores are not formed. All are readily stained, Gram-negative and non-acid-fast.

Growth Requirements. They are aerobic and facultatively anaerobic. Growth occurs between 10° and 42° C. with an optimum temperature of 37° C. They are not nutritionally fastidious and may be cultivated in simple synthetic media containing an ammonium salt and glucose.

Cultural Characters. Growth occurs luxuriantly on all media. On agar fairly large, round, low convex, greyish white, smooth, moist colonies develop. On MacConkey's medium the same type of colonies grow but are pink. The capsulated strains grow in mucoid colonies. Rough variant colonies may spontaneously occur on solid media. Good growth with uniform turbidity occurs in broth. A deposit tends to form in older cultures. Pellicle formation is not a usual feature.

Biochemical Reactions. The colon bacilli exhibit marked biochemical activity, attacking a number of sugars with the production of acid and gas (Table XII). Acid and clot are produced in litmus milk. Indole is positive. Nitrates are reduced to nitrites. Gelatin is not liquefied. A few strains are β-haemolytic; such strains are more frequent in pathological conditions than in the normal intestine.

TABLE XII

Biochemical Reactions of the Common Coliform Bacilli

	Motility	Lactose	Glucose	Saccharose	Dulcite	Salicin	Gelatin	Indole	V.P. Test	M.R .Test	Citrate-utilisation	Litmus milk
Bact. coli commune ..	+	+	+	−	+	+	−	+	−	+	−	AC
Bact. coli communior ..	+	+	+	+	+	+	−	+	−	+	−	AC
Bact. neopolitanum ..	−	+	+	+	+	−	−	+	−	+	−	AC
Bact. coscoroba ..	−	+	+	+	+	−	−	+	−	+	−	AC
Bact. acidi-lactici ..	−	+	+	−	−	−	−	+	−	+	+	AC
Bact. aerogenes ..	−	+	+	+	−	+	+	−	+	−	+	AC
Bact. cloacae ..	+	+	+	+	−	+	+	−	+	−	+	AC

Key: + = Positive results, acid and gas in the case of sugars.
− = Negative results.
AC = acid and clot.

Gas is produced more briskly and in greater amount by the aerogenes group than the coli group. The ratio of CO_2 to H_2 in the gas produced is higher with the aerogenes group, more than 2 to 1, than with the coli group in which it is approximately 1 : 1. Voges-Proskauer and the citrate-utilisation reactions are both negative, while the methyl red reaction is positive. The reverse is the case with the aerogenes group. These reactions are of considerable help in the differentiation between the coli and aerogenes groups and are used as a routine in water analysis.

Resistance. They are not markedly resistant to the common destructive agents. Heat at 60° C. destroys them in 15–30 minutes. Unlike the Gram-positive bacteria, they are relatively resistant to the bacteriostatic action of dyes.

Toxin Production. No diffusible toxin is formed. In large doses the endotoxin is toxic to experimental animals when parenterally introduced. A few strains produce a filterable haemolysin.

Pathogenicity. They form the normal intestinal flora. But under certain conditions, they become pathogenic, causing many suppurative lesions. The gall-bladder, urinary tract, appendix, peritoneum and the middle ear are the common sites. A chronic bacilluria is often due to these organisms. They may also cause septicaemia, sometimes simulating enteric fever.

Apart from their importance as casual pathogens, *Bact. coli* has interest in that it forms a very valuable index of faecal pollution of water which, in turn, indicates the possibility of contamination of water with more dangerous intestinal pathogens, like the typhoid, dysentery and cholera bacilli (p. 173).

Natural infection of lower animals with *Bact. coli* is uncommon. Diarrhoea in young calves, fowls and pigeons due to the colon bacillus has been reported; so also bovine mastitis.

The Aerogenes Group

These are more plumpy than the typical colon bacillus, often capsulated and non-motile. *Bact. aerogenes* is a typical member of this group. *Bact. cloacae* is an actively motile and non-capsulated species. This group is often subdivided into an "*aerogenes*" group and an "*intermediate*" group. But this differentiation is difficult and probably not necessary.

The members of this group form large, shiny, often effluent colonies. Abundant gas is produced in sugars, but occasionally a non-gas-producing strain may be encountered, e.g. *Bact. coli anaerogenes*. No indole is produced. Methyl red reaction is negative, but both Voges-Proskauer and citrate-utilisation reactions are positive (Table XII). Except *Bact. cloacae*, they all fail to liquefy gelatin. They are normally found on grains, in dust and soil and also sometimes in the human intestines. Occasionally they are associated with urinary infections, but their exact role in these is not certain.

Diagnosis. Direct examination of preparations from materials, whether stained or unstained, is not of much value. The isolation of the organism by the employment of differential media, like the MacConkey's or the D.E.C. (p. 106), followed by the study of the biochemical and metabolic activities, will lead to final identification. It is a common observation that certain lactose-fermenters may at times develop into pale colonies on these media. In order, therefore, to arrive at a correct diagnosis, it is necessary to put the pure cultures through fluid sugar media. In cases of suspected coli septicaemia the isolation of the organism from the blood follows the same lines as in enteric infections. Serological reactions for the diagnosis of these organisms have not been developed. Differential diagnosis of the coli group of

organisms from the aerogenes types is an important procedure in the examination of water samples (Chapter IX).

Treatment. Vaccine therapy is often useful in the treatment of infections with the colon bacillus. An autogenous vaccine is preferable to stock vaccine. Sulphanilamide compounds have also been reported upon favourably in the treatment of these infections. Streptomycin is still in the stage of investigation.

The Friedlander Group

Friedlander's bacillus is normally found in the nose, throat and intestines. It was first isolated from the lungs in lobar pneumonia. It is also called *Bact. friedlanderi*, *Bact. pneumoniae*, the *pneumobacillus* and *Bacillus mucosus capsulatus.*

Morphology and Staining. They are straight, thick rods with rounded ends, 1–2 μ long and 0·3–0·5 μ thick, non-motile, non-sporing and thickly capsulated. In culture they occur singly, in end to end pairs or in short chains. But in tissues they exist as typically capsulated diplobacilli. They are Gram-negative. Some strains are poorly stained. Special stains are required to stain the capsule.

Growth Requirements. The Friedlander bacillus is a facultative anaerobe. The optimum temperature is 37° C. with a range of 12°–43° C. The nutritional requirements are simple and the organism grows readily in ordinary media and in simple synthetic media.

They grow as large shiny or mucoid colonies on solid media. The growth in fluid media is moderate, giving rise to a slimy sediment.

Biochemical Reactions. There is no regularity in this respect. Sugar reactions are variable; some strains fail to attack lactose. Delayed production of gas in sugars is a feature with some strains, while some others may fail to form gas altogether. The production of acid and gas in glucose, mannite and maltose is fairly constant. Indole production is more uniformly negative, while the methyl red reaction is more uniformly positive. With some strains the Voges-Proskauer reaction is positive. Acid is produced in litmus milk with or without coagulation.

Types. This species does not constitute an immunologically homogeneous one. On the basis of a complex carbohydrate substance contained in the capsule, three distinct types have been

recognised—Types A, B and C. Many strains, that do not conform to these types, are classed together as a IV group. The specific carbohydrate substance of one of these types, Type B, is serologically identical with the capsular substance of Type II pneumococcus (p. 252). Under artificial conditions dissociative changes may take place readily. The rough variants are non-capsulated and lose their type specificity. As in the case of the pneumococcus, the rough antigen is species-specific. There is some evidence to show that Type A strains predominate in man and Type B strains in animals.

Pathogenicity and Toxin Production. Friedlander's bacillus is a normal inhabitant of the upper respiratory tract. It does not manufacture any exotoxin. Very rarely (less than 1 per cent.) it may cause lobar pneumonia of an extremely severe and fatal form. Alone or in conjunction with other organisms, it occurs in certain respiratory infections, suppurative conditions of the sinuses, otitis media, empyema, meningitis, cystitis and conjunctivitis. Its pathogenic role in these has not been determined. They are probably secondary invaders.

The rhinoscleroma bacillus closely resembles Friedlander's bacillus in morphological and cultural features. Bile suppresses its growth; consequently it does not grow on MacConkey's medium. It is capsulated and motile. Lactose is not fermented and acid is produced in glucose. Indole is not formed and Voges-Proskauer reaction is negative. Litmus milk is unchanged.

It causes a granulomatous disease of the mucous membranes of the nose, mouth and throat. The organism is found in the lesion, particularly inside certain giant cells called the cells of Mikulicz. Rhinoscleroma is a rare disease.

The ozoena bacillus also bears a close resemblance to the Friedlander bacillus. It is capsulated and non-motile. It is present in the condition called ozoena, but its causal relationship with it still remains doubtful.

An organism belonging to the Friedlander group has been incriminated as the cause of granuloma inguinale (infective granuloma, granuloma venereum). This disease is a chronic ulcerative granuloma, usually involving the genital, inguinal and anal regions. Transmission is assumed to be through sexual contact and the disease is included in the venereal group of diseases. Certain intracytoplasmic bacillary bodies, apparently

capsulated, occur constantly in the infected tissues and exudates. These are referred to as the *Donovan bodies* after Donovan who first described them at Madras. A bacillus of the Friedlander type has been isolated from exudates from ulcers and from affected tissues, and it has been considered identical with the Donovan bodies. The available evidence, however, is insufficient to establish this bacillus as the cause of the disease or to prove its identity with the Donovan bodies. The nature of the Donovan bodies remains yet to be unravelled. Attempts to reproduce the infection in the common experimental animals have not been successful.

The Paracolon Group

Several species of coliform bacilli possessing irregular or uncertain biochemical properties and pathogenicity are included in the paracolon group. A few species, that form both acid and gas regularly from lactose, are also included in this group by some authorities. This is open to question; their correct place appears to be in the lactose-fermenting group.

Some of the paracolon members produce only acid in lactose and in this respect they come nearer to the Friedlander group. Others, like *Bact. columbense*, are definitely inert on lactose, saccharose and salicin, exhibiting kinship with the *Salmonella* group. But, unlike the *Salmonellas*, they produce indole. The remaining members are irregular in their action on lactose or ferment it late, recalling the behaviour of the Sonne and dispar bacilli. Probably the paracolon bacilli occupy an intermediate position, and form a link, between the coliform and the *Salmonella* groups.

Of some special interest is *Bact. coli mutabile* which in its general characters appears to be closely related to the paracolon group. Though primarily a non-lactose-fermenter, it shows the capacity to give rise to lactose-fermenting variants (p. 336).

The paracolon bacilli have been isolated from various sources, such as stools of patients suffering from acute or chronic intestinal infections like enteric fever, non-specific entero-colitis and dysentery, blood in irregular febrile conditions, urine in infections of the urinary tract and even stools of normal individuals. Their pathogenicity is doubtful; some appear to be really pathogenic, but as yet there is no convincing proof and at most they can only be held under suspicion.

Bacterium Asiaticum. This is another species of undetermined taxonomic position. It is a non-lactose-fermenter. But, because of its fermentation of saccharose and production of indole, it cannot be included under the *Salmonella* group. It is motile. While it does not attack lactose and dulcite, it ferments regularly glucose, saccharose and mannite, forming both acid and gas. Indole is produced and litmus is acidified with no formation of clot. It does not liquefy gelatin. Often it has been isolated from stools, urine and blood of patients suffering from enteric-like infections, but its pathogenic role in these conditions remains still unsettled.

The Non-Lactose-Fermenting Bacteria

The non-lactose-fermenting bacteria are classified into the *Salmonella* and the dysentery groups. The former includes the enteric and the food poisoning members. The non-lactose-fermenters thus contain a large number of pathogenic species. Their action on glucose provides a convenient basis for their identification. There is one species, *Bact. alkaligenes*, of doubtful pathogenicity, which is altogether inert. *Bact. typhosum*, *Bact. gallinarum*, a pathogen in fowls, and the dysentery group of organisms all produce only acid in glucose, while the remaining bacteria produce both acid and gas. The *Salmonella* group is serologically distinct from the dysentery group.

The Salmonella Group

Salmonella is the name applied to a group of non-lactose-fermenting bacteria provisionally placed in the genus *Bacterium*. They are strict parasites and cause natural infection in man and animals. A few species are exclusively human pathogens responsible for a group of diseases known as enteric fever: these species are, therefore, classed together as the enteric group. The remaining members, about 140 species, are primarily animal and bird pathogens, but many of them may also cause infection in man which is usually of the nature of food poisoning. Hence, they are often referred to as the *food poisoning group*.

Morphology and Staining. In their morphology and staining reactions, the *Salmonella* organisms are indistinguishable from the coliform bacteria. They are readily decolorised by the Gram's techinque. Except *Bact. pullorum* and *Bact. gallinarum*,

all species are actively motile by means of peritrichate flagella. They are not capsulated and not sporing.

Growth Requirements. Their optimum temperature is 37° C., but they will grow over a wide range of temperature. They are facultative anaerobes. Their nutritional requirements are also quite simple as those of the coliform group.

Biochemical Activities. Their biochemical activity tends to be constant (Table XIII) and affords a valuable guide in the identification of species. None of them ferments saccharose nor form indole. They do not liquefy gelatin. With the exception of *Bact. typhosum* and *Bact. gallinarum*, which produce only acid in glucose, they all produce both acid and gas in this sugar. Other sugars that are employed in the identification of the *Salmonella* species are xylose, arabinose, inositol and rhamnose.

While biochemical reactions are of considerable value in the primary identification of the *Salmonellas*, of late considerable emphasis has been laid on their antigenic structure for purposes of differentiation. In fact, no other group of bacteria has been subject to so careful and thorough antigenic analysis as the *Salmonella* group. This method has also been applied extensively in the determination of the *Salmonella* species. But how far antigenic structure may be relied on for the determination of the *Salmonella* species, is an open question. Numerous species have already been defined and the process is still going on.

Antigenic Structure. The *Salmonellas* exhibit a complex antigenic structure. They bear no antigenic relationship with other subgroups of the genus. But within the *Salmonella* subgroup itself there is a certain amount of antigenic sharing between the many species with consequent cross agglutination. For example, *Bact. paratyphosum B* and *Bact. typhi-murium* (Aertrycke) share common antigenic fractions; so also *Bact. enteritidis* (Gaertner) and *Bact. typhosum* (Table XIV).

They are motile bacilli and so contain in the normal smooth state two distinct types of antigens, the somatic antigen and the flagellar antigen represented by the symbols O and H respectively. The O antigens are designated by Roman numerals. These different kinds of antigens evoke the production of separate agglutinins, also named O and H (*vide* p. 237). Spontaneous loss of flagella may occasionally happen, resulting in the production of non-motile strains which retain the smooth characteristics. Such

TABLE XIII

Biochemical Reactions of the Salmonella Group

Species	Motility	Lactose	Glucose	Saccharose	Dulcite	Mannite	Maltose	Rhamnose	Xylose	Arabinose	Inosite	Litmus Milk	Indole	Gelatin
Bact. typhosum ..	+	–	A	–	– late A	A	A	–	A	A ± late	–	A or N	–	–
Bact. paratyphosum A ..	+	–	AG	–	AG slow	AG	AG	AG	–	AG	–	A or N	–	–
Bact. paraptyphosum B	+	–	AG	–	AG	AG	AG	AG	AG	AG	AG	Alk	–	–
Bact. enteritidis ..	+	–	AG	–	AG	AG	AG	AG	AG	AG	–	Alk	–	–
Bact. typhi-murium ..	+	–	AG	–	AG	AG	AG	AG	AG	AG	AG	Alk	–	–
Bact. cholera-suis ..	+	–	AG	–	– or AG slow	AG	AG	AG	AG	–	–	Alk	–	–
Bact. paratyphosum C..	+	–	AG	–	AG	AG	AG	±	AG	AG	–	Alk	–	–
Bact. pullorum ..	–	–	AG	–	–	AG	–	AG slow	AG	AG	–	A or N	–	–
Bact. gallinarum ..	–	–	A	–	±	A	A	A slow	±	A	–	Alk	–	–

+ = motile. A = acid. AG = acid and gas. – = negative result.
Alk = alkali. ± = variable. N = neutral.

non-flagellate strains have only the somatic antigen. The O antigen, composed of lipoids and polysaccharides, represents the endotoxin. It is resistant to heat and absolute alcohol.

TABLE XIV

Antigenic Structure of the Common Salmonellas

Species	O Antigen	H Antigen	
		Phase 1	Phase 2
Bact. paratyphosum A ..	(1), II, XII	a	—
Bact. paratyphosum B ..	(1), IV, (V), XII	b	1, 2
Bact. typhi-murium ..	(1), IV, (V), XII	i	1, 2, 3
Bact. paratyphosum C. (Hirchfeld)	VI, VII, (Vi)	c	1, 5
Bact. cholera-suis ..	VI, VII	c	1, 5
Bact. typhosum ..	IX, XII, (Vi)	d	—
Bact. enteritidis ..	(1), IX, XII	gm	—
Bact. pullorum ..	IX, XII	—	—
Bact. gallinarum	IX, XII	—	—

The antigenic components in brackets may be absent.

During infection or by immunisation, both H and O agglutinins are produced, provided the respective antigens are present in the organism. But they are not produced in the same titre. While the H agglutinins are produced in very high titres, generally the O agglutinins are produced only in lower titres. The H and O agglutinins behave independently of one another and their presence in an agglutinating serum can be demonstrated and titres estimated by employing the corresponding H and O antigens. By appropriate method, these agglutinins can be selectively absorbed out from the serum by the use of the corresponding antigen, thus leaving a pure H or O antiserum—monospecific antiserum, as the case may be. The granular character of the clumps in O agglutination and the coarse flocculent character of them in H agglutination have been referred to under the section on agglutination (p. 269).

The H antigen is prepared by adding 0·1 per cent. formalin to a 24-hour broth culture of the motile strain. It may also be done by emulsifying the growth on solid media in 0·1 per cent. formol saline. The formolised suspension is not a simple H suspension; the O factor is, of course, there, but it is masked by treatment with formalin. In the preparation of the O antigen alcohol is employed to destroy the H antigen. A dense emulsion with the minimum quantity of saline is made from an eighteen- to twenty-four-hour growth on agar slope, diluted twenty times with alcohol, heated at 40–50° C. for thirty minutes, centrifuged and the sediment suspended in saline to the proper density. The growth on agar may also be directly emulsified using alcohol instead of saline. The strain employed must be smooth. It may be advantageous to use the growth on phenol agar (1–800 phenol) instead of that on ordinary agar.

In addition to the O antigen, a few of the *Salmonellas*, notably *Bact. typhosum*, *para C* and probably also *para B*, have been shown to possess another somatic antigen which is found only in the freshly isolated virulent state of the organism. It is designated the Vi or virulent antigen. It is believed to be responsible for virulence. It is a labile antigen and is present only in live cultures (p. 439).

The H antigen of many species may remain in one of two phases, the specific phase and the group phase (*specific-group phase variation*), each exhibiting more than one antigenic component in its structure (Table XIV). The H antigens in the specific phase are designated by lower case letters and those in the group phase by Arabic numerals. In the specific phase, any of these organisms reacts only with the corresponding antiserum, whereas in the group phase it also reacts with the antiserum prepared against allied species. In other words, the H antigenic components in the specific phase are peculiar to the particular species, while those in the alternate group phase display characteristics common to many species of the group. Further, some species are *monophasic* with reference to their H antigen, occurring only in one or the other phase, see Table XIV.

The terms specific and group in the *specific-group phase variation* are not quite appropriate. The antigenic components in the specific phase may not be strictly specific to the particular species concerned but may be common to a few others as well,

whereas those in the non-specific or group phase are common to many other species. Thus, the difference between them is not very sharp as the terms indicate and they are now called *phase* 1 and *phase* 2. Moreover, phasic variation has been shown to be more complex. Phase 1 itself may show two alternate phases in which the antigenic components in one phase are different from those in the other. These are referred to as the α and β phases and the changes as the *$\alpha\beta$ phase variation*. The range of phase variation does not seem to end with this and there is reason to believe that still more complicated changes may be taking place in the H antigen of some at least of the *Salmonella species* (p. 348).

Variation. The members of the *Salmonella* group are subject to spontaneous variation and with it the O and H antigens may likewise undergo transformation or be altogether lost (p. 347). The smooth motile form may become non-motile, while still remaining smooth. In this there is loss of H antigen. Another type of variation is the S–R variation in which both the motile and non-motile smooth forms may change into rough form. The changes involved in this do not occur suddenly, but several intermediate stages are observed in which the O characters are slowly shading off into the rough; in them the O antigen has not been entirely lost, revealing the rough antigen. The rough, or R antigen, is quite distinct from the O antigen. The rough variant may or may not be motile. A third type of variation is the phasic variation referred to above.

The complexities and irregularities in the antigenic constitution of the *Salmonella* bacilli clearly indicate the supreme need for keeping them in view during the practical application of the agglutination test in the diagnosis of *Salmonella* infections. It also emphasises the importance of testing the patient's serum against both O and H bacterial suspensions.

The final identification of a freshly isolated bacillus, suspected to belong to the *Salmonella* group, is based on its antigenic structure as evidenced by agglutination and agglutinin absorption tests (p. 274). The typing may be done by the slide method, employing monospecific antisera. It is obvious that a large number of such sera have to be employed. For details of these tests, bigger books should be consulted.

Pathogenicity. The members of the *Salmonella* group are responsible for the causation of two well defined disease entities

in man, the typhoid and paratyphoid fevers and an acute gastro-enteritis. The former is due to the typhoid and paratyphoid bacilli and the latter may be caused by a large number of *Salmonella* species, the most important being *Bact. enteritidis*, *Bact. typhi-murium* and *Bact. cholera-suis*. But it must be noted that this division is not sharp and that the paratyphoid bacilli may sometimes cause gastro-enteritis instead of the usual slow fever. The food poisoning bacilli, on the other hand, may often give rise to septicaemia in addition to abdominal disturbances. Besides these, the *Salmonellas* have also been reported to cause infection of a generalised or localised character. Evidence, so far as it goes, tends to show that man may act as a healthy carrier.

These organisms cause epizootics in rats, mice, guinea-pigs, rabbits and in certain birds. *Bact. enteritidis* is extremely virulent to laboratory animals, producing in them haemorrhagic enteritis and septicaemia. The Aertrycke bacillus, *Bact. typhi-murium*, likewise causes acute enteritis in guinea-pigs and pigs but only less frequently in rats. In these rodents a healthy carrier state may also be established, a point of great importance in the aetiology of bacterial food poisoning. Infected pigeons and ducks transmit the infection to their eggs. Certain animals, like cattle, pigs and horses, are readily susceptible to natural infection with these organisms. Recently, transmission of infection to man has also been traced to dogs and cats.

Bact. pullorum and *Bact. gallinarum* are members of the *Salmonella* group, displaying close resemblance to the typhoid-paratyphoid group of organisms. Both are non-motile. The former causes white diarrhoea of chicks and the latter a disease in fowls associated with severe anaemia.

The Enteric Group

The most important member of this group, *Bact. typhosum*, was discovered by Eberth in 1880, who observed it in the intestinal ulcer, mesenteric glands and spleen of persons dying from typhoid fever. In the following year, it was successfully cultivated on artificial media. Other proposed names for the typhoid bacillus are *Bacillus typhosus*, *Eberthella typhosa* and *Salmonella typhi*. Other members of this group are: *Bact. paratyphosum A*, *Bact. paratyphosum B* and *Bact. paratyphosum C*. The last

mentioned organism formerly went under the name Hirchfeld's bacillus. A typhoid-like fever may also result from infection with *Bact. cholera-suis*, particularly the monophasic *Kunzendorf* type, which is closely related to *paratyphoid C*; but it is relatively unimportant. The disease caused by *para C* and *Bact. cholera-suis* differs somewhat from that due to the other three species; in the former, symptoms due to lesions of a septic or suppurative nature, including endocarditis, often complicate the usual picture of enteric fever.

Morphology and Staining. *Bact. typhosum* consists of straight rods with rounded ends, measuring 0·5 by 2·0–3·0 microns. Long filamentous forms may occur. The organism is non-sporing, non-capsulated and actively motile with long peritrichous flagella. It is Gram-negative and not acid-fast. Not much morphological difference can be made out between the several members of the group. Hence the other bacilli are not separately described.

Habitat. The typhoid and paratyphoid bacilli are present in the intestines of individuals suffering from the infection. They may be found in their blood, the mesenteric glands and internal organs. They are always found in the excreta of patients and carriers. Outside the body, the organisms remain viable in the contaminated soil, sewage and water for varying periods, even up to a month or two. While *Bact. typhosum* and *para A* are exclusively human pathogens, *para B* has also been reported in animals; but this is only as a carrier state.

Growth Requirements. The typhoid bacillus is aerobic and facultatively anaerobic. It grows best at 37° C. but may grow at any temperature between 18° and 41° C. The pH range is also wide, 5·0–8·6, with an optimum of 6·8–7·0. The nutritional requirements of this organism are simple; it grows readily on ordinary media and in synthetic solutions containing ammonium salts and glucose. Tryptophane appears to be an essential growth requirement; whereas some strains are capable of synthesising it, others are unable to do so and have to be provided with it in the culture media.

Cultural Characters. The colonies on agar are like those of the colon bacillus, but smaller and more transparent with leaf-like surface markings. They are easily emulsifiable. Rough variant colonies may be seen side by side with the normal smooth colonies. The colonies on MacConkey's medium are smaller

and colourless. Growth in broth is abundant, giving rise to uniform turbidity. The growth of *para A* is relatively poor.

Biochemical Reactions. *Bact. typhosum* produces only acid in glucose, mannite and maltose, while the paratyphoid bacilli produce both acid and gas. Lactose and saccharose are not touched by any. An occasional typhoid strain causes slow fermentation of dulcite, producing only acid, whereas the paratyphoid strains all produce both acid and gas. Between *para A* and *para B*, the latter ferments xylose but not the former (see Table XIII).

No member of the group produces indole nor liquefies gelatin. Nitrates are reduced to nitrites and hydrogen sulphide is produced. The methyl red test is positive and the Voges-Proskauer test negative. No haemolysin is produced.

Basing on the degree of acid-production in certain sugars, like rhamnose and inosite, and on the susceptibility to the action of bacteriophage, *para B* has been recently classified into several types.

Resistance. They are easily killed by heat (55° C. for thirty minutes), drying and antiseptics; cold is resisted for long. Certain dyes, such as eosin and brilliant green, are resisted in high dilutions; so also are bile and bile salts. Hence the use of these in the preparation of selective media for isolating these organisms. In water and soil these organisms may survive for several days. They remain viable for some days in sewage and in both sea and fresh water polluted by sewage.

Toxin Production. No diffusible toxin is formed by any of these organisms. Endotoxin is presumably the toxic agent responsible for the causation of disease. The cell substance is toxic to experimental animals.

Pathogenicity. The typhoid bacillus causes in man typhoid fever and the paratyphoid bacilli paratyphoid fevers. Instead of the slow continued fever, *para C* may often give rise to acute gastro-enteritis. It is also often distinguished from the rest by the presence of septic lesions. The paratyphoid bacilli, especially *para B*, may also cause food poisoning. Animals are not naturally infected by any one of them. When administered by the oral route, experimental animals do not develop infection: but in the chimpanzee a successful reproduction of the disease has been claimed by the oral administration of massive doses of *Bact. typhosum*. When injected into laboratory animals intra-

peritoneally or intravenously in massive doses, a fatal septicaemia results and after death the organism can be recovered from the blood and tissues of the animal. Active multiplication does not seem to take place with small doses.

Antigenic Structure. The antigenic structure of *Bact. typhosum* is given on page 433. From this it will be readily seen that the organism is closely related immunologically to the *Salmonellas*. Many workers, however, doubt the validity of including it in the *Salmonella* group. Pending a decision of its correct taxonomic position, the typhoid bacillus is better placed in the *Salmonella* group with the name *Bact. typhosum*. The subjects of antigenic structure and variation have been dealt with at length elsewhere.

The Vi Antigen. Besides the H and O antigens, another important antigen associated with *Bact. typhosum* is what has been recently described by Felix and Pitt and designated the "virulence" or Vi antigen. They noticed that the freshly isolated strains of *Bact. typhosum* failed to agglutinate with the O antiserum. Such strains were also found to be particularly virulent for mice. From these and other experiments, they came to the conclusion that the difference in the behaviour of fresh strains was due to the presence of a hitherto undetected antigen which contributed to the virulence of the organism. Hence the term Vi antigen.

Practically all strains recently isolated from cases of the disease possess the Vi antigen; so also a large majority of strains obtained from carriers. As mentioned above, in the presence of the Vi antigen the organism is not agglutinated by O antiserum. The Vi antigen appears to be of the somatic type: but, unlike the O antigen, it is heat labile. It is also destroyed by phenol. The Vi strains display greater resistance to phagocytosis. By repeated culturing on artificial media, the Vi strains gradually lose the capacity to develop the Vi antigen: such strains are also found to have lost much of their virulence for mice. The strains deprived of the Vi antigen are susceptible to the action of O agglutinins.

There appears to exist a close correlation between virulence and Vi antigen. It must be pointed out that some workers do not agree with this view. The Vi antigen stimulates the production of Vi antibody which is powerfully protective as attested by the mouse protection test. Vi antibodies are present in the blood

of patients and convalescents and probably also of carriers. They act independently of H and O antibodies. There is some experimental evidence to indicate that the Vi antibody has greater protective value than the O antibody, at any rate in mice. From the above considerations the importance of the Vi antigen in active and passive immunisation will be apparent.

Bacteriophage Typing. This method is based on the existence of bacteriophages which act specifically on the Vi antigen. By propagating a phage serially on a particular Vi strain of the organism, it becomes highly adapted to that strain. Thus, different races of bacteriophage endowed with a high selective affinity for different groups of the Vi forms of the organism can be reared up. The selective lytic property of such a specially adapted phage remains stable, making a stable classification of the Vi strains possible. Although bacteriophages lytic to O strains exist, the sharing of O antigen among the *Salmonellas* render any bacteriophage method based on the O antigen useless for the differentiation of types. The technique of typing is very elaborate and requires considerable experience. Employing this method, eleven main types of Vi strains of *Bact. typhosum*, designated A, B, C, D, etc., have been distinguished. They are fairly stable. Typing is of great value in epidemiological investigations: it enables to correlate cases and trace out the original source of infection.

Enteric Fever. Enteric fever is a world wide disease, more prevalent in the tropics and subtropics than in the higher latitudes. Two factors that are vitally concerned with the prevalence of enteric fever are the progress of sanitation and the protection of water supply. Where these are not efficient, as in backward countries or during military campaigns, enteric fever prevails in epidemic form. When we realise that infection enters the body through food and drink, it can be readily appreciated how a close interrelationship of individuals constituting the host population can be productive of conditions favourable for the easy transmission of infection. Where the conditions are wide spread, the resulting infection is also perforce wide spread, *i.e.* in epidemic intensity.

Though cases may occur throughout the year there is a marked seasonal variation in its incidence, the monsoon months being the peak period in this country. No age is exempt, but the

maximum incidence is among the adolescents and the young adults. Typhoid fever is far more serious and fatal than paratyphoid fevers; the latter are also of shorter duration and less liable to complications. Fatality increases with age; it is also high below five years of age.

The type incidence varies in different localities. This is particularly applicable to paratyphoid infections. *Para A* is much more common in India than *Para B*, whereas the reverse is the case in Europe. As contrasted with other enteric fevers, *paratyphoid A* infection is peculiar in its wave-like incidence. *Para C* is of very restricted occurrence anywhere.

Active cases and carriers form the source of infection. The results of systematic agglutination test indicate that the frequency of infection is far more than the frequency of declared cases. Hence carrier spread must be more common than case spread. Infected matter from the intestines of cases and carriers should finally reach the alimentary tract of the healthy susceptible man. It is obvious that it can happen only through the mouth and that water and food are the vehicles of infection. The organisms are excreted in the faeces and, less commonly, in the urine, and contamination of food and drink with these infected materials, which serve as the connecting link, should precede the actual transfer of infection from the sick to the healthy. The existence of conditions that favour this pollution bespeaks of the poor general sanitary level of a community. Faecal pollution of the water supply, either directly or through sewage, is the commonest mode of spread. Like cholera, enteric is also an important water-borne disease. Enteric bacilli may not live outside the body over long periods, but they survive in water long enough for transmission to occur. This method of transmission usually results in large-scale epidemics. Pollution of milk and ice cream is another method and the typhoid bacillus can survive at low temperature fairly long (p. 173). Infected shellfish is yet another, though rare, agency of spread in some countries: it gets the infection from sewage-polluted water. Through contaminated hands of carriers or of attendants on cases to food is still another mode of transport of infection. Flies also act as portal carriers. Their intestines too may get infected, when transfer of infection to food would occur also through the insect's excreta. Again, vegetables, such as radish, lettuce and other leafy vegetables,

that are likely to come in contact with contaminated water or other infectious materials, may set up fresh infection; some of them are usually taken uncooked.

Carriers. Enteric carriers form an important source of the spread of infection. They serve to transmit the infection in their own localities or to other localities where they move to. There are three types of carriers: the convalescent carriers, chronic carriers and healthy or contact carriers. All the three types are responsible for transmitting the infection. The majority of convalescent carriers get rid of the infection rapidly in a few weeks. About 2–4 per cent., however, fail to do so and remain as permanent or chronic carriers. Paratyphoid carriers are fewer than typhoid carriers. Chronic carriers are the most important and most dangerous transmitters of enteric fever. Healthy contacts, such as hospital orderlies and nurses, also become temporary carriers. The carrier may be of the faecal type when the condition is very likely dependent on a persistent infection of the gall-bladder, or, very rarely, the urinary type as a result of the infection lurking in the pelvis of the kidney or in the urinary bladder. A combined faecal and urinary carrier is not also very uncommon. Due to the greater facility for spread, the urinary type is equally, or more, dangerous as the faecal type. Women carriers are more common than men carriers. There appears to have some correlation between the frequency of women carriers and the frequency of gall-stones. Unlike the Shiga carrier, the enteric carrier is apparently healthy. The excretion of bacilli from the carriers is intermittent, making repeated examination imperative for a correct diagnosis.

The site of infection is the small intestine. The possible sequence of events occurring in the course of infection appears to be somewhat as follows: The organism gains entry thorugh the mouth, in food or drink. The normal acidity of the stomach is sufficient to destroy it, but the stomach may be empty or its contents too diluted, so that the organism escapes unscathed into the duodenum and jejunum. On reaching the intestine, the bacilli do not directly settle down and start disease. They attack the lymphoid tissue, which is thus the primary site of infection, and thence pass on into the blood stream through the mesenteric glands and the lymphatic route, causing bacteraemia, and are then carried all over the body. The bacteraemia is, however,

transitory as it is soon followed by the removal of the organism from the blood by the reticulo-endothelial system. Small foci of infection are then set up in the liver, gall-bladder, spleen, kidneys, bone marrow and other organs where the bacilli multiply in large numbers. Subsequently, from these foci there is an overflow of the organisms into the blood stream, starting the phase of secondary septicaemia, during which they are excreted in large numbers by the bile into the intestines. The organism displays a selective affinity for the lymphoid tissue and during the reinvasion of the gut, infection of the Peyer's patches and the solitary lymphoid follicles of the intestine occurs followed by rapid multiplication of the organism, leading to an acute inflammatory reaction, hyperplasia and infiltration with mononuclear cells. Necrosis and sloughing follow with the formation of characteristic ulcers. This is the specific lesion of the disease and it is preceded by septicaemia and generalised infection. Changes similar to those occurring in the Peyer's patches are also found in the mesenteric glands.

Rash is due to minute cutaneous bacillary emboli. Haemorrhage, caused by erosion of a blood vessel at the base of an ulcer, and perforation are only accidental complications and do not form part of the essential pathology of the illness. The spleen is usually enlarged and congested. Suppurative and inflammatory lesions may occur in other parts of the body as complications and sequelæ and the organisms are found in them in large numbers. Cholecystitis, cystitis and periostitis are none too infrequent.

The invading organism is present in the blood stream from the onset of symptoms and may continue to be present there for varying periods during the course. Being an intestinal infection, the bacilli may be present in the faeces at any time in the course of the disease; but towards the end of the second week and thereafter, they may be recovered from the faeces with increasing frequency. They are also excreted in the urine, but with less frequency. The bacilli are present in the rose spots. They are, of course, present in large numbers in the intestinal ulcers and lymphatic tissue, the mesenteric glands and spleen. Stained sections of liver and spleen show localised masses of bacilli. The gall-bladder is one of the persistent sites of infection, a fact of great importance from an epidemiological point of view. The

bone marrow and, rarely, the pelvis of the kidney are other sites.

Clinically, the incubation period, which ranges from two to three weeks, is from the day of infection to the day of the second invasion of the blood stream, and this secondary septicaemia with reinvasion of the gut marks the onset of actual disease. The stage of acute inflammation and hyperplasia takes about a week and that of necrosis and sloughing probably another week or ten days.

Relapses may occur during convalescence, but they are milder and of shorter duration with a very low mortality rate. Relapses are caused by the organism reinvading the blood from the secondary foci. Probably, antibodies formed as the result of the primary attack, while insufficient to prevent relapses, are nevertheless sufficient to make them mild. The milder nature may also be accounted for by a ready and energetic response of the tissues to the reinfection of blood which is of the nature of secondary stimulus (p. 241).

Immunity. It is not clear whether man possesses any natural immunity against enteric fever. Among people equally exposed to the risk of infection, some escape it altogether, some get the disease in a mild form, while others contract a serious type, often succumbing to it. How far endemic immunity is the underlying factor in causing this wide divergence in the degree of resistance, is not quite apparent.

Antibodies are incited early in the disease and agglutinins, bacteriolysins and immune opsonins begin to appear in the blood in about five to seven days after the onset of the illness. With the progress of the disease, their concentration increases in most cases and parallel with it the organisms progressively disappear from the blood. But it is also an observed fact that the presence of a high concentration of antibodies does not always protect the patient from death. Recovery from an attack of enteric fever is followed by a fair amount of immunity lasting for some years: but second or even third attack may occur. This immunity is not necessarily associated with a high titre of serum antibodies; in fact, the titre rapidly falls off to a negligibly low level in a short time and persists at that. The basis of protection, therefore, is to be sought for in the sensitised tissues readily and vigorously responding to any possible infection and aborting it.

Active immunity developed as the result of one type of enteric fever does not protect against any other type. Persons recovered from typhoid fever may still get any of the paratyphoid fevers in future and *vice versa*. Whether any cross immunity would have developed in them in virtue of antigenic relationship is not known.

Diagnosis. The isolation and identification of the infecting organism from materials obtained from the patients form the most dependable test in the bacteriological diagnosis of enteric fever. Though not equally reliable, the Widal test, done on the blood of the patient, is also of great practical value.

Materials that may be used for culture are the blood, faeces, urine or the duodenal contents. The frequency with which a positive culture can be obtained from any of these depends upon the stage of the disease at which the material is examined. Blood culture is the method of choice in the first week of the disease. During this period it yields 80–90 per cent. success. After the first week, the organism begins to disappear from the blood with the result that the success of blood culture steadily falls, so that by the third week a positive blood culture is obtained only in about one-third of the cases. The disappearance of bacteraemia, it may be noted, is associated with a progressive rise in the agglutinin content of blood. However, attempt should always be made to isolate the organism from the blood, whatever be the stage of the disease at which it comes under observation. A bacteraemia in the late second or third week is not a happy sign. Ordinarily, blood culture is a relatively simple matter. About 10 c.c. of blood should be withdrawn from a convenient vein under aseptic precautions and inoculated into 50–100 c.c. of a suitable liquid medium. By gentle shaking the blood should be mixed well with the medium. A 0·5 per cent. sodium taurocholate broth is the one usually employed and is quite satisfactory. Sterile ox bile (5 c.c. blood + 10 c.c. bile) obtains favour with some workers. When clotted blood is received, remove the clot to the sterile medium, break it up and culture (clot culture) and preserve the serum for the agglutination reaction. This is not a satisfactory method, but superior results have been claimed for it. The method of isolating the organism from the primary fluid culture is given in the next paragraph.

It is customary and also advisable to proceed with the study of the biochemical activities of the isolated organism. But their final identification rests mainly on the specific agglutination test. For this purpose, specific agglutinating sera, high titre sera, are prepared in the rabbit and kept ready for use. The test may be done by the slide agglutination method or by the macroscopic method; details of these are given elsewhere (p. 272). It should be remembered that freshly isolated strains of typhoid bacilli, containing the Vi antigen, may often give negative agglutination reaction with the specific O serum, as the Vi antigen inhibits the interaction between the O antigen and its antibody.

The isolation of the offending organism from the faeces may become necessary if the blood culture has been a failure or when a clearnace test is called for before discharging patients. It is also of supreme importance in the detection of carriers. Next to blood, cultivation of faeces gives the greatest percentage of positive results. The success in isolation from stools depends upon many factors, such as the number of the causative organisms present in the motion, the suitability of the medium employed, the amount of inoculum used for culture and the efficiency of the technique. The number of bacilli present in the faeces depends upon the stage of the disease. They are discharged most abundantly towards the end of the second week and during the third week. Unnecessary delay in the culturing of stools and urine after removal should be avoided and care should be taken to preclude the possibility of any antiseptic coming in contact with them. Such delays and contaminations also tend to reduce the percentage of success. In case delay is inevitable, as when a specimen has to be sent to a distance, before sending one volume of motion should be mixed with two volumes of a 30 per cent. glycerol in 0·6 per cent. saline. Delay should likewise be avoided after the specimen reaches the laboratory. If the motion is solid it should be emulsified with sterile saline before inoculation. The use of a selective fluid medium as a primary step would greatly facilitate isolation as it would inhibit the growth of the rich bacterial flora, normally contained in the faeces, without interfering with the growth and multiplication of the enteric organisms. Brilliant green bile broth or broth containing sodium tetrathionate or selenite forms a very satisfactory medium for the primary enrichment culture. After the enrichment

process, a suitable solid medium is employed for differentiation. MacConkey's agar is the differential medium usually employed. Recently, several other selective media, such as the bismuth sulphite agar of Wilson and Blair or the D.E.C. medium (p. 106), have been claimed to be superior to MacConkey's agar.

Excretion of these organisms in the urine is far less frequent than in the faeces; but when it happens, it is in large numbers and ordinary methods of cultivation prove easily successful. In the isolation of the organism from the faeces or urine, one single negative result should not be relied on.

Widal Reaction. The agglutination reaction when applied in the diagnosis of enteric fever is called the Widal reaction. In doing the test we are searching for the presence of the specific agglutinins, and their concentration, in the patient's serum, by employing known strains of the different species of the enteric group. A positive result of this test is very helpful but not conclusive, except in a minority of cases.

The technique of the Widal reaction has been described elsewhere (p. 272). The test may be done by the microscopic or the macroscopic method. The latter is more reliable. The microscopic, or the slide method, is resorted to only rarely; it is more commonly employed as a preliminary test in the identification of newly isolated enteric organisms. A negative slide test is not a conclusive proof against the organism being any member of the enteric group.

The enteric organisms are not equally prevalent everywhere, the type distribution differing in different localities. Hence, the choice of antigen employed in any laboratory depends upon the type of infection prevalent in that locality. It may be recalled that *Bact. typhosum* has more or less universal distribution. *Para A* is more prevalent in the eastern countries, like India, while *para B* is more prevalent in the European countries. *Para C* is much restricted in its occurrence. In many laboratories, *Bact. typhosum*, *Bact. paratyphosum A* and *Bact. paratyphosum B* are always employed in the test with one other *Salmonella* species peculiar to the locality. The strains employed in the preparation of antigen should be the smooth form and of known high agglutinable potency. All these species are motile containing both the somatic and the flagellar antigens and so the test is to be conducted with both O and H suspensions. Further, the suspen-

sion must have a certian density. As mentioned elsewhere, the H antigen is made by formalinising and the O antigen by treating bacterial suspension with absolute alcohol (p. 434). In all laboratories where the Widal reaction is done as a routine, a battery of suitably prepared and standardised antigens of both types are always kept ready for use.

The Vi antigen stimulates the production of Vi antibodies. But it has been found that, whenever the Vi agglutinins are produced, the H and O agglutinins are likewise formed. Hence the Vi antigen is not usually included in the Widal reaction.

For the following reasons both H and O antigens should be employed in the test. The O agglutinins develop earlier than the H agglutinins and if O antigen is not included in the test, the corresponding O agglutinins will be missed and the chance of making an early diagnosis will be reduced. Sometimes only the O agglutinins are formed. It is but very rarely that the opposite phenomenon of only the H agglutinins developing obtains in typhoid fever, though it may occur in paratyphoid infections. The presence of the O agglutinins, as evidenced by a positive agglutination reaction with the O antigen, may also show up that some other organism of the typhoid group and sharing the same common O antigenic fractions as the typhoid bacillus may be responsible for the fever under investigation, thus providing a clue for search in a different direction. This will not be possible if the H antigen alone is used. Further, the anamnestic response, sometimes associated with certain infections, usually affects only the H and not the O antibodies. Furthermore, an O agglutination test is far more helpful in the diagnosis of enteric fever with a previous history of protective inoculation.

There are several sources of fallacy and considerable experience is necessary for a correct interpretation of the results. When reading the results, the possible occurrence of the prozone phenomenon should be kept in mind (p. 269). Again, what titre can be taken as diagnostic, is influenced by several factors and no hard and fast level can be laid down.

The sera of normal persons may contain agglutinins for enteric organisms. Such positive individuals constitute only less than 5 per cent. These "normal" agglutinins, when present, are usually of the O type and they occur only in low titre, less than one in fifty (Felix). Others give the limits of such normal

agglutination as: *Bact. typhosum*—H, 1 : 30 and O, 1 : 50; *Bact. paratyphosum B*—H, 1 : 30 and O, 1 : 50; *Bact. paratyphosum A*—H and O, 1 : 10. These are the limits observed in Britain, but they vary in different localities. They are probably higher in endemic areas.

The stage of the disease at which the test is conducted is another factor. Agglutinins usually begin to appear between the 5th and the 7th day but may do so earlier or later. During the first week, only less than 20 per cent. give positive results and then in not more than 1 : 50 titre. The titre of the reaction then goes on increasing up to a maximum reached by about the end of the third week and then tends to fall, markedly at first, but slowly later. Generally speaking, while blood culture proves positive in about 20 per cent. of cases at this period, Widal reaction gives 80–100 per cent. Therefore, in the first week a negative reaction may be the result or when a positive reaction is obtained it may be only in a low titre. Nevertheless, it is of importance to perform the test at this period as a rising titre in a subsequent test is of great value in the diagnosis. A persistent low titre agglutination throughout the course of the disease generally points to a negative diagnosis. The fresh appearance of a low titre agglutination in the later stages is very suspicious.

In those who have had protective inoculation with typhoid-paratyphoid vaccine, it has been said that agglutinins produced are of the H type, whereas in the natural disease both H and O agglutinins are formed. Recent work tends to disprove this and several workers have shown that O agglutinins are produced in vaccination, though not to the same titre as the H agglutinins. At any rate, six months after inoculation, it has been found that only a low titre of H agglutinins for all the organisms, not more than 1 : 200, and a much lower titre for O, less than 1 : 50, are found. The probable presence of such residual agglutinins in patient's blood again shows the importance of repeating the test and demonstrating a progressively increasing titre before establishing a positive diagnosis.

The phenomenon of non-specific stimulation, the anamnestic reaction, should also be kept in view when interpreting the results (p. 240). An attack of influenza or some other acute infection in an immunised individual may cause a rise in titre of the

existing agglutinins. It seldom goes above 1 : 100. Usually such non-specific reactions are not seen in the case of O agglutinins (Felix).

It is obvious from the above facts that no particular titre can be laid down as definitely diagnostic; a rising titre, as shown by repeating the test once in three days, is the most valuable procedure. This is particularly important in the vaccinated individuals. However, certain titres have been accepted as significant and should lead to a repetition of the test. These should be well beyond the range of normal reactions in individuals without previous protective vaccination and beyond the range of residual titres in vaccinated patients (*vide supra*). In the previously unvaccinated patients, a positive reaction of 1 : 40 H.T. and 1 : 80 O.T. is suggestive of typhoid fever and anything above these is diagnostic. Usually a very much higher titre is obtained after the first ten days. These titres when obtained with *para B* antigens are equally applicable to *paratyphoid B* infections. But it should be noted that the O agglutinins are not infrequently poorly developed in *para B* infections. In *paratyphoid A*, lower titres than these are said to be sufficient for diagnosis. Bu infection with *para A* organism often produces as high a concer tration of agglutinins as the typhoid fever. High titres with bo H and O antigens are obtained even in the early stages of enteric fever occurring in those who have had protective immunisation. In them a titre of 1 : 200 with O antigen is suggestive of typhoid and *para B* infections and 1 : 400 diagnostic. A high titre with H antigen, 1 : 1280 or higher, is significant even in vaccinated persons. The diagnosis of *paratyphoid A* may be done with lower titres than those for the typhoid and *para B* bacilli.

A high titre of H agglutinins, with O agglutinins absent or present in low titre, suggests either recent prophylactic vaccination or possibly non-specific stimulation from some other infection. The opposite state of a high O agglutinin content, with H agglutinins absent or present in low titre, is strongly suggestive of the disease even in previously immunised subjects. Frequently, sera from typhoid patients also show agglutination of *paratyphoid A* and *B*. This is due to the group relationship, as can be readily seen from a glance at the antigenic chart given on page 433; it is only in relatively low titre and should not cause serious difficulty in arriving at a correct diagnosis.

Diagnosis of Enteric Carriers. The isolation of the carrier organism from the faeces or urine of a person constitutes the surest proof that he is a carrier. The excretion of organisms from carriers is intermittent and so one negative result alone has no value. At least three consecutive examinations should be conducted before a negative report is given. On the ground that the organisms are likely to be present in the greatest concentration in the bile and in the contents of the small intestine, the administration of a dose of calomel overnight followed by a saline purgative in the morning and collection of the second or third stool for examination have been advocated. Culture of the specimen should be performed by two different methods, the ordinary method and an enrichment method as described above. The examination of urine is to be pursued on the same lines as in the diagnosis of acute cases. The collection of the duodenal contents for the recovery of the organism has also been recommended.

The Widal reaction is not of great help in detecting carriers. It is positive in the majority of carriers. But a positive reaction may also be obtained in persons who are not carriers. Besides, a negative Widal reaction by no means excludes the carrier state. The Vi agglutination test is considered to be of particular assistance in the detection of typhoid carriers. It is reported that the majority of carriers have in their blood Vi antibodies.

In the Vi agglutination test, the antigen employed is a bacterial suspension prepared from the growth of a known Vi strain on solid medium. Such a strain should react only with the Vi agglutinin and not with the H and O agglutinins. A concentrated suspension may be prepared and preserved for use. It deteriorates on keeping and should not be used after two months. Dilutions of the test serum are made as in the ordinary test in 1 c.c. volumes. Serum from a haemolysed specimen is unfit for the test. One drop (0·05 c.c.) of the concentrated suspension is then added to each tube. The tubes are incubated at 37° C. for two hours and left at room temperature overnight. The results are read by the aid of a hand lens on the following day. A titre of 1 : 10 is considered suggestive in cases of suspected typhoid and should lead to a repetition of the test. As in the ordinary Widal reaction, a rising titre is more conclusive. The same titre, 1 : 10, is also regarded as suggestive in the case of suspected carriers.

Treatment. A typhoid antiserum, prepared by immunising horses with living typhoid bacilli, has been tried with little encouraging results. A Vi antiserum, recently prepared, has been claimed to yield greater success. It protects rats against typhoid infections, while O antiserum does not. Immune sera containing both O and Vi antibodies should theoretically be of value by neutralising the toxic antigens and by helping to destroy the bacilli. Such sera have been prepared and their limited trial would seem to testify to their superiority over others.

Specific Prophylaxis. It consists in immunising individuals with typhoid-paratyphoid (T.A.B) vaccine. This is prepared from selected smooth strains of T, A and B, possessing maximum virulence. The culture should be exposed to heat and chemicals as little as possible. Heavy growth, obtained on solid media, is washed off with sterile physiological salt solution and killed by heat at 60° C. for thirty minutes. As a preservative phenol or tricresol is added in proper concentration (p. 145). The vaccine is then standardised so as to contain in 1 c.c. 1,000 million typhoid bacilli and 750 million each of *para A* and *para B*. The dose of this vaccine is 1 cubic centimetre. Better protection is obtained by giving two doses than one dose, the first one 0·5 c.c. and the second 1 c.c., 7 to 10 days apart. The dose is reduced in children proportional to their weight. It is administered subcutaneously. Slight local and general reaction may follow in some individuals. Intracutaneous method has been advocated by some people who claim that it evokes better immunity response and is less liable to cause general reaction. Three doses of 0·1, 0·15 and 0·2 c.c. have been recommended for this.

The prophylactic value of protective inoculation against enteric fever has been fully established, particularly by the results of mass immunisation of the fighting personnel; and now there is universal agreement on this point. Protection once conferred lasts for a year. Hence, vaccination should be repeated every year, especially when there is the threat of an outbreak of epidemic and in field conditions. Protection does not completely safeguard against future infection; recurrence may happen, but such cases are rare and milder and less fatal than those among the unvaccinated.

The method of sterilising the T.A.B vaccine by heat, as mentioned above, is unsound in principle. The degree of heat

employed destroys the Vi antigen. So also the phenol used in the subsequent preservation. Such a vaccine lacking in the Vi antigen will not be able to stimulate the production of Vi antibodies. For this reason, both killing and subsequent preservation should be done in alcohol. A vaccine prepared in this way has been found to incite the production of Vi antibodies. Hence, the immunising efficiency of it should be decidedly greater; this awaits practical confirmation.

Bilivaccine has not the same prophylactic efficiency as the parenterally administered T.A.B vaccine. The former has the advantage that it is orally administered. It is prepared in ten times the concentration of the ordinary vaccine. After killing, the vaccine is dried, usually mixed with starch and put up in capsule form. Three doses are given, each one hour before breakfast on three successive mornings. The administration of three-fourth of a grain of bile salts about one hour before the vaccine is recommended in the belief that they would ensure better absorption.

General Prophylactic Measures. In countries, like Britain and U.S.A., where sound general sanitary measures form part not only of their public health scheme but also its practice, the incidence of enteric fever has been very largely controlled. General measures include efficient sanitary arrangements, particularly in the matter of sewage disposal; protection and purification of water supply; protection of milk and other vehicles of infection, such as ice cream and shellfish; the effective disposal of infected materials from the patient and a vigorous campaign against flies. Carriers are the most potent agents in the spread of the disease and also form an effective link between epidemics. Hence, their detection and control constitute another important prophylactic measure. It is extremely difficult to get rid of the carrier state. Ordinary measures, such as vaccine therapy and chemotherapy, have proved unavaling. Cholecystectomy in the case of the faecal carrier is a very effective measure. However, it is of restricted applicability. Surgery obviously has no use in the case of urinary carriers. The more practical method of not employing carriers as cooks and servers in such trades as concerned with the manufacture or handling of food should be adopted.

BACTERIUM ALKALIGENES

The systematic position of *Bact. alkaligenes* has not been decided. As it displays certain general properties of the *Bacterium* genus, it is provisionally placed in it.

Bact. alkaligenes is a motile, Gram-negative bacillus quite inert on sugars. Its motility is due to the presence of peritrichate flagella. Plenty of alkali is produced during growth and their colonies on MacConkey's plate are surrounded by bleached zones due to the diffusion of alkali into the medium. It gives rise to a characteristically brown growth on potato. Litmus milk is rendered deep blue in two or three days. The organism may be found in normal faeces. It has been isolated from the blood and urine of patients suffering from infections of the enteric type. Its pathogenicity is still a matter of doubt.

A long, thin and slightly curved organism, somewhat resembling *Bact. alkaligenes*, but provided with lophotrichate flagella, has been described under the name of *Vibrio alkaligenes*. Its relationship with the former is not known.

THE FOOD POISONING GROUP

Bacterial Food Poisoning. Organisms mainly associated with food poisoning belong to the *Salmonella* group. In morphology and general characters they resemble the enteric group of organisms. Biochemically, they are all inert to lactose and saccharose, but produce both acid and gas in glucose and a number of other sugars (Table XIII). Indole is not formed by any of them. In metabolic activities and resistance, they resemble the enteric group. No extracellular toxin is formed by any of these organisms. Growing in food materials, they produce a powerful endotoxin; it is stable, withstanding a temperature of 100° C. for thirty minutes.

In a general way, the term food poisoning may be employed to denote all types of trauma caused by unwholesome food. The ill effects may be due to contamination with the organisms of cholera, enteric or dysentery, which go under the name of food-borne diseases; to the intrinsically poisonous nature of food, as in the case of certain poisonous fish, toadstools, atriplex or ergot; to certain chemicals, like lead and arsenic; and to bacteria and bacterial products. But the term is restricted by convention to the last mentioned group. Food allergy is not

food poisoning, for in this the abnormality is not in the food but in the allergic person.

Bacterial food poisoning is due to the ingestion of contaminated food, uncooked or imperfectly cooked. The condition is due either to the actual infection by the contaminating organism, the *infection type*, or to the presence of preformed bacterial toxin in the food, the *toxic type*. Investigations have proved that the condition is not due to ptomaine poisoning, as was once believed. Putrescine, cadaverine, methylamine and others, which are formed as the result of bacterial decomposition of protein and collectively called ptomaines, are not toxic when administered by mouth (p. 217). Attempts to reproduce the disease in man by feeding with the culture filtrate of the food poisoning bacilli have not succeeded; therefore, the condition is assumed to be due to infection. In food poisoning, particularly the infection type, organisms are present in the intestines. Frequently, they also spread to the blood stream inducing bacteraemia or septicaemia.

The infective organisms are chiefly: *Bact. enteritidis* of Gaertner, *Bact. typhi-murium*, *Bact. cholera-suis* or the suipestifer bacillus and less frequently the paratyphoid bacilli. Besides these, several other members of the *Salmonella* group, such as *Bact. stanley*, *newport*, *derby*, *oranienburg* and others, have been reported from different regions as the responsible agents. Besides the *Salmonellas*, other organisms, like *Proteus*, members of the coli group, streptococci, staphylococci and even dysentery bacilli, have been incriminated in various outbreaks. This is not surprising as food forms an excellent pabulum for the rapid growth and multiplication of micro-organisms.

The toxic type is due to the ingestion of preformed toxin in prepared food, such as canned or preserved food. Exotoxins, like the enterotoxin of staphylococci and the botulinum toxin, produce acute intoxication (see under the respective organisms).

Meat, milk, fish or eggs are the materials usually affected. Vegetables and cereals are very much less frequently and fruits only very rarely affected. Pork and beef are probably the commonest agents. Preparations like cheese, sausages, sandwiches, pies and similar canned and preserved meats often serve as vehicles. The infected food is taken imperfectly cooked or uncooked. Obvious evidence of contamination, be it remembered, may often be wanting either in look, smell or taste.

Infection of meat and other food materials may occur as the result of disease in the animal, sick cattle or pigs, or by exogenous methods. Mention has been made before that some of the *Salmonellas* are important pathogens of these animals. Extraneous infection may be by a human carrier transmitting it during slaughtering of animals or during preparation of the food. Rodents, such as rats or mice, harbouring infection either in an active or quiescent condition, may also carry the infection before or after preparation. Natural infection of rodents with *Salmonellas* are all too common. Egg is infected as the result of infection of ovum in the oviduct of the infected parent bird.

Food poisoning occurs in small outbreaks and as isolated cases. Reports of food poisoning are not so frequent in this country as in some of the western countries. Food poisoning is more common in the hot summer months as a warm temperature favours rapid multiplication of the offending organisms. The incubation period is very short, may be even a few hours in the toxic type. The symptoms are those of acute gastroenteritis associated with marked toxaemia; septicaemia is often present; even the meninges may be invaded causing meningitis. The disease is of short duration, the symptoms usually subsiding in two or three days with complete recovery. The mortality rate is very low, about 1 per cent. The condition is more fatal in the young than in the old.

Diagnosis. History is very essential for a correct diagnosis. The clinical features are also very helpful. A laboratory diagnosis is made by investigating the remnants of the suspected food, and vomit, faeces and blood from the sick person. Attempts should always be made to isolate the organism from these materials. The injection of a portion of the left off food into mice or guinea-pigs should be performed. These animals are very susceptible to the *Salmonella* organisms. If the animals get sick, attempt is made to isolate the organism from them. Efforts should likewise be made to isolate the organisms directly from the food, vomit and stools. Blood culture from patients may also yield positive results. In all these, the procedure is the same as in the case of isolating enteric organisms.

Agglutination test with the patient's serum affords little or no help in the diagnosis during the acute stage due to the short duration of the disease. It may be done during convalescence

if required for epidemiological or legal purposes. Standard suspensions should be used in the carrying out of the test. The identification of the isolated organism is not easy. The study of biochemical reactions, no doubt, will help (*vide* Table XIII). But only serological tests can confirm the identity. A reference to the table on page 433 will show that the Gaertner bacillus and the typhoid bacillus have the same somatic antigen. There is also close serological resemblance between *Bact. typhi-murium* and *Bact. paratyphosum B*, the only antigenic difference between them being that in their specific phase they possess different flagellar antigens. Again, *Bact. paratyphosum C* and *Bact. cholera-suis* contain common antigenic components. For identification, therefore, direct agglutination tests have to be supplemented by absorption tests described elsewhere (p. 273).

Treatment and Prophylaxis. Except in botulism, no specific treatment is necessary. An antiserum is available for the treatment of botulism. Prevention is very important. The control of meat at every stage till its consumption reduces considerably the chances for contamination. The condemning of infected animals before slaughter and a thorough system of meat inspection are some of the important points. The extermination of rodents in places where food industry is carried on should be undertaken. The non-employment of carriers in dairies and in food industry should be insisted. The use of fresh meat wherever possible, eschewing of damaged articles of food, thorough cooking and pasteurisation of milk are other measures.

The Dysentery Group

This group includes several species that cause one type of acute colitis in man called bacillary dysentery. A similar clinical syndrome may be caused by a number of other parasitic agents, chief of which is *E. histolytica.* The first member of the dysentery group to be discovered was *Bact. shigae* by Shiga in 1898 during an epidemic of dysentery in Japan. Two years later the same organism was discovered in Germany by Kruse and so *Bact. shigae* is also referred to as the Shiga-Kruse bacillus. About the year 1900, Flexner and Strong obtained a different type from the stools of dysentery cases in the Philippines, now called *Bact. flexneri.* Several other types were subsequently

described from various parts of the world, but their claim to separate identity or causal relationship to the disease had not been accepted for a long time. As a result, for several years, much confusion prevailed in the nomenclature and identity of types. The dysentery bacilli are strict parasites and are found only in the lesions they cause. Outside the body, they may live but for short periods.

Morphology. These organisms resemble the typhoid bacillus in morphology and staining characters. They are 2–4 microns long. Shorter and even coccobacillary forms are not uncommon. With the exception of the Newcastle bacillus, which is feebly motile, all are non-motile. They are non-sporing, non-capsulated and Gram-negative.

Growth Requirements. The dysentery bacilli are aerobes and facultative anaerobes. The optimum temperature is 37° C. They are not fastidious in their nutritional requirements and will grow readily on ordinary media. Nicotinic acid appears to be an essential requirement with a few species and in synthetic solutions they have to be provided with this substance.

TABLE XV

Biochemical Reactions of the Dysentery Group

Species	Motility	Lactose	Glucose	Saccharose	Dulcite	Mannite	Rhamnose	Xylose	Sorbite	Litmus milk	Indole	M.R.
Bact. shigae ..	–	–	A	–	–	–	–	–	–	sl A	–	–
Bact. ambiguum ..	–	–	A	–	–	–	A	–	A ±	sl A	+	–
Bact. flexneri ..	–	–	A	A late ±	–	A	A ±	–	A ±	sl A to Alk	±	±
Bact. sonnei ..	–	A late	A	A late	–	A	A	–	–	A late clot	–	–
Bact. newcastle ..	+	–	A g	–	A g late	A ±	?	sl A ±	–	sl A to sl Alk	–	+
Bact. alkalescens	–	–	A	–	A	A	A	A	A	Alk	+	+
Bact. dispar .	–	A late	A	A late	–	A	A	A	A	A late clot	+	+

Key: – = Negative results. sl = slight. ± = variable.
g = small amount of gas. A = acid. Alk = alkali.

Biochemical Activity. The dysentery bacilli are less active biochemically than the coli group. They form only acid, but not gas, in certain sugars. The Shiga and Schmitz types ferment only glucose. Others ferment mannite and a few more sugars. Hence the primary classification of the dysentery bacilli into mannite-fermenters and non-mannite-fermenters (Table XVI). Sonne's and the dispar bacilli ferment lactose and often saccharose very slowly. Certain Flexner strains also cause late fermentation in saccharose. Slight amount of gas is produced in glucose and dulcite by the Newcastle bacillus. Litmus milk is rendered slightly acid, but some strains of the Flexner group turn it alkaline after the initial acid phase. In the case of *Bact. alkalescens*, the reaction is throughout alkaline. The production of indole by the dysentery bacilli is not uniform. This serves as an important differential feature. The Schmitz bacillus produces indole, but not *Bact. shigae*. *Bact. dispar* produces indole, thus differing from *Bact. sonnei*. *Bact. alkalescens* forms indole, but *Bact. newcastle* is negative. Indole production varies among the flexner strains (Table XV).

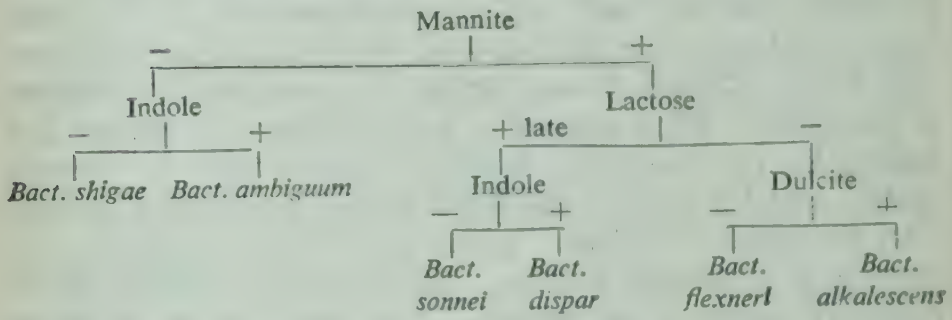
Resistance. Heat and other adverse agencies easily destroy them. They are less resistant to the bacteriostatic action of dyes than the enteric group of organisms.

Classification. From their action on mannite, dysentery bacilli are primarily classified into mannite-fermenters and non-mannite-fermenters. The former include the Flexner group, Sonne's bacillus, the Newcastle bacillus, *Bact. alkalescens* and *Bact. dispar*, each serologically distinct. It is doubtful whether the last two species are pathogenic. The non-mannite-fermenters include *Bact. shigae* and *Bact. ambiguum*. In addition to these types, several unidentified organisms are met with in bacillary dysentery. They are grouped as atypical dysentery bacilli. Further classification of the dysentery organisms is based on their serological reactions. *Bact. shigae* is a homogeneous group; so also *Bact. ambiguum* and *Bact. sonnei*. A point to be remembered of Sonne's bacillus is that it may go rough soon under artificial conditions. The Flexner group is a heterogeneous one, the members exhibiting a complex antigenic structure. Andrewes and Inman classified it into five types: V, W, X, Y and Z. Boyd has recently regrouped them, on the basis of his studies, into V, W, Z, 103, P 119 and 88, designated also by Roman numerals:

Type I, II, III, IV, V and VI respectively. According to him Type X is only an incomplete variant of Type Z and Type Y

TABLE XVI

Classification of the Dysentery Bacilli



(Hiss and Russell) the common degradation product of the specific types, containing only the complex group antigen without any type-specific antigen; that is, the old Type Y is only a laboratory product. The Newcastle bacillus is very likely identical with Type 88, with which it is serologically related. It would seem from his work that there are at least six separate specific antigens representing six distinct serological types and in addition there are many common non-specific or group antigens. These group antigens are responsible for the cross agglutination occurring between the types. This work awaits confirmation. As stated above, several more species resembling the dysentery bacilli in some respects have been isolated from cases of dysentery, but their exact relationship to the disease still remains undetermined.

Toxin Production. Of all the dysentery bacilli, the Shiga bacillus is the most toxic. They all produce powerful endotoxins. Only the Shiga type elaborates in addition an exotoxin; the latter is peculiar in that it does not diffuse out of the bacterial cell so readily as the classical exotoxins. Hence, some authorities regard it only as an endotoxin. It can be obtained free of cells by filtering a week-old broth culture. It is destroyed by heat at 75°–80° C. in one hour. It is very toxic to rabbits and mice. Administered intravenously into the rabbit, it causes diarrhoea and collapse, ending fatally. It consists of two components, a neurotoxin and a histiotoxin. Immunisation of horses with it produces a specific serum of high antitoxic value. In the case of the other dysentery bacilli, endotoxin is the only toxic factor.

The smooth somatic antigen (the endotoxin) of *Bact. shigae* is a polysaccharide-lipoid-protein complex; it is highly toxic and powerfully antigenic.

Pathogenicity. Laboratory animals fed with cultures of dysentery bacilli do not develop any disease. But, when fed with massive doses or intravenously administered, rabbits, mice and to a less degree guinea-pigs develop haemorrhagic enteritis or peritonitis and die. There is, however, no similarity between these lesions and the natural disease in man. With a Shiga culture the animals may also develop muscular paralysis if they survive long enough.

The dysentery bacilli, with the exception of *Bact. alkalescens* and *Bact. dispar*, cause dysentery in man. They are also related to the summer diarrhoea of infants and children (*vide infra*). The pathogenic role of *Bact. alkalescens* has not been fully established and *Bact. dispar* appears to be non-pathogenic.

Bacillary dysentery is prevalent in all countries and climates, but it is more common and more severe in the tropics and sub-tropics than in the temperate regions. Bacterial dysentery is far more common than the protozoal. About 80 per cent. of dysentery in India is said to be bacterial and the rest mostly amoebic. Dysentery due to *Balantidium coli* is very rare. Certain intestinal flagellates, particularly *Giardia intestinalis*, are also occasionally responsible for mild cases of dysentery and diarrhoea. About 55 per cent. of bacterial dysentery is due to the Flexner type and the rest due to the Shiga and Sonne bacilli. Bacterial dysentery, particularly that of Shiga origin, is primarily an epidemic disease. Sporadic occurrence is also common. It is also associated with large collections of people, as in barracks, jails, hospitals and asylums. The so-called asylum dysentery is only a type of bacterial dysentery. It lingers in the asylums because of the insanitary condition usually prevailing there.

Dysentery shows seasonal variation and in India its peak is during the heavy monsoon months. This period also corresponds to the months of highest fly population. In colder countries it is more common during summer. The mode of transmission is through food and drink, contaminated directly from active cases or carriers or indirectly by flies. There is undoubtedly a close relationship between flies and dysentery epidemics, indicating the former's role in transmission. Contaminated water does not

seem to play so important a role in the spread of dysentery as it does in typhoid fever.

Bacillary dysentery is an acute infectious disease in which there is acute inflammatory and often necrotic changes in the mucosa of the large intestine, rarely extending to the last part of the ileum. A clinically identical condition is caused by *Entamoeba histolytica* and certain other intestinal protozoa. Like cholera, bacillary dysentery is a local infection confined to the primary lesion in the gut and associated with general manifestations from systemic toxaemia. Generally, beyond the focal mesenteric glands and the intestinal mucosa, there is no bacillary invasion and only very rarely, if at all, are the liver and spleen invaded. That is to say, unlike in typhoid fever, there is no blood infection in dysentery.

The incubation period is generally short, about two to three days. From mild diarrhoea all grades of severity occur. Generally, the Shiga dysentery is a more severe type, the mortality rate being about 20 per cent. Complete recovery from it is a rather prolonged process and tendency to chronicity is a great danger, as it leads to life-long colitis and invalidism. The immunity against the dysentery bacilli is feeble and one attack, even when completely cured, does not protect against subsequent attacks.

Carriers. Carriers form a great source of danger to the community, as they play an important role in the transmission. Convalescents become rapidly bacillus-free, in seven to ten days. About 2–3 per cent. persist to harbour the infection even after three months. They are the permanent or chronic carriers. Some authorities also believe in the existence of healthy carriers. The discharge of the organism from the carrier is intermittent; the Shiga carrier is a more constant excretor than the Flexner carrier. The dysentery carrier is of the faecal type; the urinary type does not occur as there is no systemic infection in dysentery. The clinical difference is also noteworthy. The Shiga carrier is ill and is practically an invalid, while the Flexner carrier is comparatively well.

Diagnosis. *Entamoeba histolytica* causes a clinical condition exactly similar to bacillary dysentery and a differential diagnosis between them is extremely important, as the specific treatment of these two types is entirely different. The bacteriological diagnosis of dysentery rests almost solely on an examination of

the patient's faeces. The examination of blood for the presence of the specific agglutinins may rarely help.

The faeces should be collected in suitable sterile containers and despatched carefully and immediately after it is voided. No urine or antiseptic should be allowed to mix with it as the dysentery bacilli are killed by these. The mucus portion should be selected for examination. A rectal swab may sometimes be more convenient, particularly when examining children.

The naked eye appearance and reaction may be suggestive. The motion in bacillary dysentery is small, mucoid, blood stained or purulent, with no faecal smell and showing alkaline reaction. In amoebic dysentery, it is large, foul smelling, mixed with blood, mucus and faeces and acid in reaction. A loopful of the specimen is mixed with a loopful of saline and examined under a cover-slip with the one-sixth objective. An abundant cellular exudate, consisting mostly of pus cells, red blood corpuscles and epithelial cells, is very characteristic of bacillary dysentery. Macrophages, large mononuclear cells with relatively large nuclei and often vacuolated, may be present, simulating immobilised amoebae. The rather healthy appearance of the red cells, arranged in regular rouleaux formation and the absence of the pathogenic amoebae and Charcot Leydon crystals are other suggestive indirect evidences of a bacillary aetiology. In amoebic dysentery, amoebae are seen and cellular exudate is scanty or absent. The only other dysenteric condition in which cellular exudate may rarely be obtained is giardiasis. But in this, the characteristic vegetative or cystic parasite is present in large numbers.

The isolation of the organism followed by its identification is the only sure evidence. Dysentery organisms are present in the faeces from the very commencement of symptoms, their number progressively decreasing. They are rapidly destroyed in the faeces: hence, cultivation should be proceeded with without avoidable delay. But if delay is unavoidable, one part of faeces is mixed with two parts of 30 per cent. glycerol in 0·6 per cent. saline. The chances of successful isolation decrease with the duration of the illness. In the first three days, about 70–80 per cent. of specimens yield positive results; after this the success rate rapidly declines.

The methods employed for the isolation of the dysentery bacilli from faeces are essentially the same as those employed in

the isolation of the typhoid bacillus. The mucus portion from the faeces is directly plated on a selective solid medium, MacConkey's or preferably D.E.C. After twenty-four hours of incubation the non-lactose-fermenters are isolated and studied with reference to the morphological and biochemical reactions. The final recognition of the type is established by the agglutination test, employing the specific sera which are available against all the types. When the Flexner type is suspected, a polyvalent serum should be used as there are many serological races in it. When testing for Sonne's bacillus, freshly isolated culture should be employed because the organism undergoes dissociation rapidly. The Schmitz type can be identified by employing the homologous serum.

Agglutination test with the patient's serum has only a very limited value. Agglutinins appear five to seven days after the onset of the disease, but diagnosis would have been made by this time by the cultural method. The procedure for the test is the same as in the Widal reaction; but it should be remembered that dysentery bacilli, being non-motile, contain only the O antigens. In testing for the Flexner group, standard suspensions of all types should be employed and for Sonne's type, a freshly isolated strain.

When interpreting results, the possible presence of natural agglutinins to the dysentery bacilli and the development of group agglutinins in the patient's blood should both be kept in mind. The titre of natural agglutinins varies in different places. It may be 1 : 20 against Shiga's bacillus, 1 : 100 against the Flexner type and 1 : 50 against *Bact. sonnei.* Again, *Bact. shigae* stimulates the formation of a high titre of anti-Flexner agglutinins in the patient's blood, whereas the opposite seldom occurs. Infection with the typhoid bacillus is also sometimes found to stimulate the production of agglutinins against the Flexner group. The following minimum titres, therefore, may be taken as diagnostic: 1 : 40 against the Shiga antigen, 1 : 150 against the Flexner group in the absence of agglutinins against the Shiga and the typhoid bacilli and 1 : 100 against Sonne's bacillus. Generally, agglutinins in dysentery may completely disappear in three months. Sometimes they persist, especially in carriers.

The isolation of the infecting organism from carriers is very difficult. Enema fluid or, better still, scrapings from ulcers taken

through a sigmoidoscope should be examined. One negative result is not conclusive. Serology may prove more useful in the detection of carriers.

Prophylaxis. The general principles of prophylaxis are the same as those employed against enteric infections. The non-employment of carriers as cooks and servers is important; so also fly control.

Active immunisation may be tried. Vaccines are prepared in the same way as the T.A.B vaccine. The Shiga vaccine is very toxic and should be used carefully. The Flexner vaccine should include all the serological strains. Bilivaccine has the advantage that it is orally administered, but is even less effective than the heat-killed vaccines. Dysentery vaccines have not proved satisfactory and are seldom used. Superior results have been claimed for a Shiga vaccine specially prepared by treating the culture with 0·5–1·0 per cent. formalin for two weeks at 37° C.; it is less toxic and large doses can be given without risk.

Treatment. Therapeutic sera are prepared from horses. Refined and concentrated sera are available. The serum treatment gives better results in Shiga infection than in others. In infection with the Flexner type, a polyvalent serum should be used. The Shiga serum is both antibacterial and antitoxic, while sera against other types are only antibacterial. The serum should be administered as early as possible. Large doses, 50–100 c.c. of the unconcentrated serum, should be given intramuscularly, and in serious cases intravenously, and repeated if necessary at intervals of twenty-four hours until a favourable response is forthcoming. Treatment with bacteriophage is of doubtful value.

Chemotherapy. Sulphanilamide group of drugs has a definite place in the treatment of bacillary dysentery. After their advent, serum therapy has receded to the background. The sulpha compounds should be given in large doses. Sulphaguanidine, sulphathiozol and sulphadiazine are the compounds usually employed.

Summer Diarrhoea

Summer diarrhoea is a dysentery-like illness occurring in children during the summer and early autumn months. It may occur sporadically and in epidemic form. Its incidence is much less in breast-fed children than in artificially fed children.

Children of the poorer classes suffer more. A close relationship between the prevalence of flies and the incidence of summer diarrhoea has been shown to exist; that accounts to some extent the seasonal incidence. Besides, the hot season also favours the rapid multiplication of bacteria in food and milk.

Its aetiology is not definitely known. It is not unlikely that many of these cases are due to infection with organisms of the dysentery group. But it is also likely that other organisms are also responsible for its causation. Different organisms, such as *Bact. flexneri*, *Bact. sonnei*, *Prot. morgani*, the paratyphoid bacilli, *Ps. pyocyanea*, streptococci and *Cl. welchii*, have all been reported as causative from different countries.

Examination of the stools is the most valuable procedure in establishing the cause. Prevention is by adopting those general prophylactic measures employed against the incidence and spread of dysentery. Special care should be paid in the feeding of children. The control of flies should receive particular attention.

CHAPTER XXI

VIBRIO AND SPIRILLUM

Vibrios are short, curved, rigid rods motile by means of a single (or rarely two or three) polar flagellum. They are aerobic and facultatively anaerobic and usually Gram-negative. Several species have been described, some of which are pathogenic to man, animals or birds.

Of the many species that constitute the genus *Vibrio*, only one is pathogenic to man. It is the causative organism of cholera discovered by Koch and variously known as *Vibrio cholerae*, *Spirillum cholerae* and *Vibrio comma.* In connection with the search for the causative agent of cholera, several closely resembling species have been described by other workers in the intestine of man, in water and other situations. As they differ immunologically from *V. cholerae* and as their causal relationship to cholera is doubtful, they have been placed together under the name *Paracholera group.* The El Tor strains, first isolated at the quarantine station at Tor on the Red Sea coast, also come under this group. Another species often encountered in the intestinal contents in cholera nostras and cholera infantum is *Vibrio proteus*, described by Finkler and Prior. Several other vibrios pathogenic to animals but not to man have been identified, for example *Vibrio metchnikovi* and *Vibrio fetus.* Many more saprophytic and free living species have been found occurring in water, manure, infusions, carious tooth and other situations.

V. cholerae, commonly called the cholera bacillus or the comma bacillus of cholera, was discovered by Robert Koch in 1884 during his investigation into the aetiology of cholera in Calcutta and Egypt.

Ecology. The organism is found in the intestinal contents of patients and carriers and for a very short time in water and soil contaminated with their excreta. There is no proof for the suggestion (d'Herelle) that the non-agglutinating vibrios found

in the tanks and wells in the endemic areas are degenerate saprophytic variants of the true comma bacillus.

Morphology and Staining. The cholera vibrios are slender, slightly bent, rigid rods from 1·5–3·0 microns long and 0·4–0·6 micron broad, often resembling a comma. The ends are rounded or slightly pointed. The cells are arranged singly, in S shaped pairs or short spiral chains. Smears from intestinal contents display the characteristic shape and arrangement like shoals of fish in a stream. In culture they may be very short and may soon lose the typical bent shape. Involution forms too are very common. The cholera bacillus is very actively motile by means of a single short terminal flagellum, executing a darting or scintillating type of motility. It neither develops a spore nor a definite capsule. The organism is readily stained by the ordinary coal-tar dyes; it is Gram-negative and non-acid-fast. Dilute carbol fuchsin is the stain of choice.

Growth Requirements. The cholera vibrio is strongly aerobic and therefore a thick surface growth on liquid media is a common feature. It is also facultatively anaerobic, but growth is poor in the absence of oxygen. The optimum temperature for growth is 37° C. with a range of 14°–42° C. The organism tolerates a high pH, up to 9·6, whereas even slight acidity is injurious to its growth. The reason why a healthy stomach is a powerful protection is therefore apparent.

V. cholerae grows readily in all media. As mentioned above, a strong alkaline reaction, up to pH 9·6, favours growth. Dunham's peptone water, adjusted to a pH of 8·4, is a suitable medium and in this growth takes place in six to eight hours. The employment of a high alkaline medium is, therefore, the usual method for the isolation of the cholera bacillus. In such a medium the colon bacillus and other intestinal associates either do not grow at all or take a much longer time to grow. Dieudonne's alkaline blood agar, the D.E.C. medium and Aronson's medium are the selective solid media in common use (p. 107).

Cultural Characters. On agar plate growth occurs in twenty-four hours as round, low convex, transluscent, smooth, glistening colonies with entire edge and about the size of the colonies of the typhoid bacillus (q.v.). The colonies are soft and easily emulsifiable. A frequent feature is the occurrence of a mixed type of colony in which one part is white and opaque, while

the rest is of the usual clear type. A gelatin stab culture shows a streak of growth along the needle track after twenty-four hours, which is soon followed by liquefaction. The organism grows luxuriantly in broth, producing moderate turbidity, thick surface pellicle and slight flocculent deposit.

Biochemical Activity. The organism ferments glucose, saccharose, maltose, mannite and mannose with the production of acid without gas. It does not attack lactose, dulcite and arabinose. After a number of days lactose may be fermented. The production of acid in saccharose and mannose and the non-production of it in arabinose differentiate *V. cholerae* from most of the paracholera strains. Litmus milk is rendered alkaline at the top and acid at the bottom. It is not coagulated but peptonised. Indole is produced and nitrates are reduced to nitrites. The cholera-red reaction is due to the presence of both indole and nitrites. The addition of a few drops of concentrated sulphuric or hydrochloric acid to a twenty-four- to forty-eight-hour old culture in peptone water causes the development of a rose pink colour due to the formation of nitroso-indole. This is a constant character of *V. cholerae*. But it is not peculiar to the cholera vibrio; any organism that reduces nitrates to nitrites as well as forms indole will give this reaction; many other vibrios too give a positive reaction.

The organism liquefies coagulated serum and gelatin. It produces ammonia and hydrogen sulphide. A yellowish brown pigment is elaborated in two or three days when grown on potato slopes rendered alkaline. No haemolysin is formed, a point of difference from the El Tor type.

Resistance. The cholera vibrio does not display great resistance to the various adverse influences. Heat at 55° C. kills it in ten to fifteen minutes. Drying destroys it in two or three hours. The organism may survive in clean water up to sixteen days but in cesspool perishes in twenty-four hours. It keeps alive for some days at freezing temperature and because of this the indiscriminate use of ice during epidemics is risky. The organism may also keep alive for several days on fresh vegetables, fruits and moist linen. It is rapidly destroyed by chemical disinfectants.

Antigenic Structure. All the known vibrios are placed under two groups, A and B. Group A contains the cholera and

cholera-like vibrios and Group B all the rest. Being flagellate organism, the vibrios possess both H and O antigens. As they are often monoflagellate, the H antigen is not so pronounced as in the *Salmonellas*, nor is it so significant. It is non-specific and common to all the cholera and cholera-like vibrios. On the basis of the somatic antigen, the Group A vibrios are classified into six immunological subgroups, each containing a separate O antigen (Gardner and Venkataraman). Subgroup 1 contains the cholera vibrios and some El Tor strains. Subgroups II to VI contain all the cholera-like vibrios, including most of the paracholera group. The somatic antigen of the cholera vibrios contains at least three complex polysaccharides which are probably responsible for specificity. Besides the major O antigen, common to the species, minor O antigens appear to be present in the somatic complex of *V. cholerae* and on the basis of this three subgroups have been defined. Two of these correspond to the Inaba and Ogawa strains mentioned below. According to this scheme, therefore, *V. cholerae* has a non-specific H antigen, a major specific O antigen and at least two minor O antigens. The major O antigen is common to all strains of *V. cholerae* and also to some El Tor strains. In addition to these, a non-specific protein constituent, common to all cholera-like vibrios, has also been described. The report on the antigenic structure of the El Tor vibrios is however conflicting. Recent work in this country would seem to indicate that antigenically these organisms are somewhat different from the true cholera vibrios (Taylor and others).

On the basis of cross reaction, earlier workers had defined three types of *V. cholerae*, the Inaba, Ogawa and Hikojima, all capable of causing cholera. Of these, the Inaba is the most and Hikojima the least common types. In this country, the Inaba is the most prevalent type.

Variation. The virulence of the cholera bacillus is rapidly lost by subculture. In artificial culture the S–R variation may readily occur. It may be hastened under the influence of bacteriophage, lithium chloride and other agents. The usual train of correlated changes, including changes in the antigenic structure and loss of virulence, accompanies dissociation (p. 343).

Toxin Production. *V. cholerae* produces no soluble toxin. But old broth cultures are powerfully toxic. It is due to the endotoxin derived from the dead and disintegrated bacterial body.

This toxic factor accounts for the profound toxaemia and collapse characteristic of cholera. It has been reported that when it is administered intravenously, the endotoxin produces in experimental animals diarrhoea and pathological lesions in the intestines and kidneys, which are not unlike those produced in cholera.

Pathogenicity. *V. cholerae* causes cholera in man. Its aetiological relation to cholera has been proved by the results of laboratory accidents as well as of a deliberate experiment. Challenging the validity of Koch's claim, Pettenkofer and Emmerich swallowed some broth culture of the organism isolated by Koch. The former developed a mild and the latter a serious attack of cholera, showing plenty of cholera vibrios in their stools, thus satisfying Koch's postulates. Also, there have been many instances of accidental laboratory infection followed by disease, passing plenty of *V. cholerae* in their stools.

Only man is naturally infected. Experimentally, cholera can be reproduced by feeding very young rabbits. But in older rabbits, guinea-pigs, mice and monkeys the organism fails to produce any harm when given by mouth. The gastric juice may be acting as an effective barrier. The intraperitoneal inoculation in these animals is followed by haemorrhagic peritonitis, septicaemia and death in one or two days.

Cholera. India was the home of cholera from ancient times and from here several pandemics had spread to the outside world. The first recorded instance was in 1817 when it invaded several countries in Asia. There were several pandemics after this. The second pandemic, 1826–37, spread not only to Asia but also to Europe by 1832 and even to distant America, causing several thousands of death. It was during the fifth pandemic, 1883–96, that Koch discovered the causal agent of the disease. At the present time, in addition to India, cholera is endemic in many other countries, such as South China, Persia, Arabia, South East Russia and the West Indies. In India the endemic centres are Lower Bengal (the delta of the Ganges) and Assam, north-eastern part of the United Provinces, Puri in Orissa and South East Madras. In these places cholera always exists and year after year breaks out into epidemics of varying severity during certain parts of the year. The disease when it occurs in non-endemic areas is imported and the infection dies out after the epidemic.

Cholera is most prevalent during the hot weather and under famine conditions. Pandemics and epidemics follow the trade routes and similar modes of human intercourse. Large congregations of people, as in fairs and festivals, form the ideal condition for the propagation of the disease. With the speeding up of transport facilities, the spread of epidemics has also been rendered rapid. A study of these conditions gives valuable data to forecast the occurrence of epidemics two to three months in advance.

The source of infection is the acute case or the convalescent carrier and the infectious material is their excreta and vomit. Cholera is mainly a water-borne infection. The survival period of the cholera vibrios in water has been found to be five days with rare instances of obtaining a positive culture up to sixteen days (Taylor). Food, milk, vegetables and fruits may also serve as vehicles. Pollution of water takes place by the careless disposal of the infectious material—nightsoil or vomit. Food and other articles of diet may receive infection through the infected hand either of a person attending on a case or of a carrier of unhygienic personal habits, thus completing the faeces-hand-food chain. Flies also may transmit infection to food.

Carriers. The balance of evidence is against the existence of chronic cholera carriers. Investigations conducted in this country show that convalescent carriers usually become free of the specific vibrio by the end of five days with a maximum of 13 days. The healthy or contact carriers excrete the comma bacillus in their stools. Their number is small in the beginning of an epidemic but in the course of it may increase to 6–8 per cent. of the total population of the locality. *V. cholerae* has been isolated from their stools up to five days and very rarely up to nine days from onset of the first connected case (Taylor). The contact carrier himself may develop the disease very rarely, but his importance is in the dissemination of infection and the starting of fresh epidemics in new areas.

Cholera affects the lower half of the small intestine. The organism multiplies in the lumen of the gut. They are found in the lumen of the glands, in between the epithelial cells and even in the basement membrane, but not beyond. It has been reported that the organism may invade the gall-bladder on its passage down to the intestine. This seems very doubtful. Blood infection does not occur. In cholera there is no attempt at inflammation. Due

to the extremely toxic nature of the endotoxin, there is severe irritation, formation of copious mucus and denudation of the epithelial coating. These form the flakes in the characteristic rice water stool which is highly alkaline in reaction. The functions of the liver and kidney are profoundly depressed. Profuse diarrhoea, vomiting, marked dehydration, suppression of urine, cramps and toxaemia are the characteristic features.

The mortality rate in cholera is very high, varying from 30–80 per cent. in different epidemics and in different years. The yearly death rate in British India including Burma from 1877–1936 has been estimated. It has ranged between 3·7 per thousand of the population in 1900 and 0·2 per thousand in 1932.

Immunity. All people who are exposed to infection and swallow the cholera germ do not develop the disease. Whether natural resistance to *V. cholerae* has anything to do with it, is not clear. The efficiency of the gastric secretion, which readily kills the comma bacillus, may have a potent influence in determining infection.

Laboratory animals, actively immunised with the cholera vibrio, yield highly potent immune sera. Such sera contain specific antibacterial antibodies, such as agglutinins and bacteriolysins. Their role in protection is not clear. The agglutination test is specific and it has now replaced Pfeiffer's test in diagnosis as well as in the final identification of the cholera bacillus (p. 284). Standard sera for the agglutination test are prepared against the specific O antigen. As mentioned in a foregoing paragraph, the H agglutinins are unimportant and non-specific. In chalera the agglutination test is not of much diagnostic utility due to the short duration of the disease and the rather late appearance of these antibodies.

It is said that recovery from an attack of cholera protects against subsequent attacks. The immunity conferred is antibacterial. Regarding the duration of this immunity or its exact nature nothing definite is known. From the effects of vaccination and from other limited observations, we may surmise that it may not last more than a year or two.

Diagnosis. The laboratory diagnosis of cholera is relatively easy. The isolation of *V. cholerae* from materials obtained from the patient followed by its identification is the surest method. Motion, vomit and rectal swab from patients and after death

intestinal contents from a ligatured portion of the gut are the materials available.

Vibrios showing the characteristic motility and shape may be seen in large numbers in a wet preparation. Few epithelial cells may be present, but pus cells are conspicuously absent. Examination of a thin smear, made preferably from a flake of mucus and stained with dilute carbol fuchsin, would confirm the above findings. In the stained smear the morphology is better made out than in the wet preparation and often there is in the former "the fish against the current" arrangement of the bacilli.

For culture the material is inoculated into a tube of peptone water of pH 8·4 and also on a Dieudonne's, Aronson's, or D.E.C. plate. The alkaline peptone water serves as an enrichment medium and in it growth occurs on the surface in six to eight hours. In order to save time, from the surface growth transplant is made into a fresh plate. By next day discrete colonies would have grown on all the plates. After confirmation by colony appearance and smear examination, subcultures are made from a few likely colonies into as many peptone water tubes. At this stage a slide agglutination may also be done with emulsion made from the colonies on the plate and the specific test serum, preferably monospecific. By this procedure a provisional diagnosis can be given in about twenty-four hours and in clinically typical cases during epidemics this is sufficient. The isolated pure cultures are further studied morphologically and biochemically. The final identification, however, is made by the specific agglutination test with the homologous O serum. This test is confirmatory and has now replaced the bacteriolysin test of Pfeiffer which used to be the official test prior to the development of the specific agglutination test.

The agglutination test with the patient's serum is not of practical help in the diagnosis of cholera, as agglutinins develop only after five or six days of the onset of disease by which time the patient is either dead or well on the road to recovery.

The examination of carriers, water and milk for the presence of *V. cholerae* is unusually difficult in contrast to that of acute cases. It is due to the fewness of the bacilli present in the specimen as well as to the presence of saprophytic vibrios simulating the true cholera vibrio. To get over the difficulty, the enrichment method is to be followed. In this a large amount

of the inoculum is mixed with a correspondingly large quantity of the alkaline peptone water and incubated. After six hours, a few cubic centimetres of the culture is removed from the surface into fresh peptone water, repeating this procedure, if necessary, two or three times. Then, any vibrio that may be present is isolated, as mentioned above, and identified. The confirmatory test should be more rigid in this case and should also include pathogenicity tests on mice or guinea-pigs. Some people even regard it necessary to find out, when a vibrio is non-agglutinating, if it cannot be converted into an agglutinating one by serial passage through the mouse or guinea-pig.

Serological tests may be of some value in the detection of carriers. They may have agglutinins and lysins in their blood. A positive agglutination reaction in a titre of 1 : 100 is significant.

Prophylaxis. Protective measures against cholera should be taken well before the expected outbreak of an epidemic. They include general measures aimed at the collective protection of the community and the specific protection of the individual by cholera vaccination.

Vigilant care should be taken against the introduction of a case into a locality. Any suspected case should be immediately put under rigid quarantine. Other measures are the early detection and diagnosis of the first case, thorough sterilisation of excreta and other infected materials, protection of water supply, protection of food and insistence on efficient cooking of all articles for consumption during epidemics. Bacteriophage, choleraphage, as a general prophylactic has been employed. The addition of it into all sources of drinking water at the outset of an epidemic is the method. Its effect has neither been proved nor disproved beyond doubt.

First Ferran and later Haffkine employed live attenuated cultures for specific immunisation. It was soon discarded due to the occurrence of large number of deaths. A heat-killed vaccine was subsequently used by Kolle and later Haffkine. The vaccine is now prepared by heating a saline suspension at 55° C. for one hour. Two doses at intervals of 7–10 days are given. The first dose contains 4,000 million and the second 8,000 million vibrios. It may cause some local and general reaction. The immunity developed lasts probably for a year or two. The heat-killed vaccine is now being replaced by a phenol-killed vaccine

which is claimed to be superior to the former. In this method the vibrios are killed by the addition of 1 per cent. phenol to a saline suspension made from a 24-hour agar growth and without the application of heat. The concentration of phenol is then reduced to 0·5 per cent. The mode of immunisation is as in the case of the heat-killed vaccine. Bilivaccine by mouth, though favourably reported on, is not as efficient as the heat-killed vaccine (p. 453).

Therapy. An antibacterial serum prepared from the horse has been tried with disappointing results. Choleraphage has been used as a therapeutic agent. Drachm doses are given every thirty minutes till the condition improves (De and Chatterjee). In cholera this powerful lytic agent is found in the intestine, especially towards the end of the disease and during convalescence. The natural termination of the disease has been suggested to be due to the destructive action of phage on *V. cholerae*.

The sulphonamide group of drugs has been reported upon favourably in the treatment of cholera both from this country and China. But these results have yet to be confirmed by more extensive and properly controlled field and laboratory investigations.

The Paracholera Group. Vibrios closely resembling the true cholera bacillus sometimes occur in mild cholera-like epidemics and in sporadic cases. Similar organisms are also frequently found in the stools of proved cholera cases. Natural waters, both in the endemic and epidemic areas, likewise show the presence of such organisms. They are not agglutinable by the specific O serum of *V. cholerae*. Their causal relationship with clinical cholera has not been proved. Their biological relationship with the cholera vibrio has also not been fully investigated. Recent work in this country would tend to show that they are not choleragenic and that their presence in the stools of cholera or cholera-like cases is only a reflection of their presence in water and other external sources, which is all too frequent in the tropics. Many of the members included in the paracholera group, for example the El Tor type, produce a soluble haemolysin which sharply differentiates them from the true cholera vibrios. The paracholera vibrios are not serologically homogeneous but are composed of several serological races, each with a distinct O antigen. They seem to have a common H antigen among themselves and with *V. cholerae*, but their O antigen is different from that of the latter.

Other pathogenic Vibrios. Several species are known to be pathogenic to animals, birds and fish. *V. metchnikovi* was first isolated from a cholera-like disease in fowls. It closely resembles *V. cholerae* morphologically and biochemically. But in its capacity to coagulate milk, high virulence for guinea-pigs and fowls, extreme invasiveness and in serology, it differs from the cholera bacillus. *V. fetus* causes infectious abortion in sheep and cattle. It is rather difficult to cultivate this organism. It is biochemically inert and has no pathogenicity for guinea-pigs.

SPIRILLUM

Spirilla are rigid spiral rods with a varying number of preformed coils. They are usually motile by means of a tuft of flagella occurring at one or both poles. They differ from the spirochaetes in that the latter have loose flexible coils which may straighten out after death. They are generally Gram-positive. They are found in water and putrid infusions. The only species of medical interest is *Spirillum minus*, the causal organism of rat-bite fever.

Spirillum Minus. *Sp. minus* was first discovered in man by Futaki and co-workers in 1916. It is present in the blood and tissues of the patient. It is also a natural parasite of rats which act as healthy reservoir hosts. Rat-bite fever has been reported from many parts of the world. It appears to be common in Japan. The disease is not very uncommon in this country.

Morphology. The organism is short, thick with tapering ends. It is 2–5 microns long and shows 2–3 coils. Longer forms may be encountered, up to 9 microns with as many spirals. The structure of the organism is rigid and the coils are permanent. *Sp. minus* is very rapidly motile by means of one or more flagella at one or both ends. It is readily stained by the Romanowsky stains. It has not been cultivated.

Pathogenicity. The parasite causes a relapsing type of fever in man, sometimes lasting up to a year. Man gets the infection through the bite of infected rat. About 3 per cent. of rats have been found to be carriers. Very rarely the bite of cats and ferrets also may transmit the infection to man. *Sp. minus* is not present in the saliva of infected rats. Very likely it is present in the tissue about the nose and gums (Knowles).

Guinea-pigs and mice are susceptible. Other organisms, one of which is an actinomyces, have also been incriminated as

the cause of rat-bite fever. This is probably based on secondary infections that commonly occur through the primary wound (p. 207).

Through bite the organism is deposited by the rat into the wound. Usually the wound soon heals leaving a scar. At the end of 15 or 20 days, fever starts with the breaking out of the scar into a painful discharging ulcer. This is associated with inflammation of the lymphatics draining the area, inflammation and enlargement of the focal lymph glands and often skin eruptions. The fever is of a relapsing type, lasting for about three days and recurring after another three or four days. It may go on for three or four months or even up to a year. The organism is chiefly a tissue parasite, but it also circulates in the blood during the pyrexial period. At the commencement of the apyrexial interval it filters off again into the tissues.

Diagnosis. The laboratory diagnosis of rat-bite fever depends upon the finding of the organism in the discharge from the wound or juice aspirated from the focal glands. These materials in positive cases show the organism in fairly large numbers. Though present in the peripheral blood during fever, the number is too small to be easily detected. Owing to the exceedingly rapid nature of the movement of *S. minus*, the dark ground method of examination is not a good procedure. But the detection of the organism in smears, stained by Lieshman or Giemsa, may be more successful.

Animal inoculation is done as follows. At the height of fever about 2 c.c. of blood from the patient are injected intraperitoneally either into a young guinea-pig or mouse. Both animals are susceptible. The guinea-pig develops the disease after an incubation period of about ten days. The parasite is present in the peripheral blood in fairly good number. The infected guinea-pig develops a progressive febrile disease and dies in four or five months, or later still, with typical lesions. It should be remembered that mice, rats and guinea-pigs are naturally prone to infection with spirochaetes; this is a source of error that should be guarded against when doing the animal inoculation test.

Prophylaxis and Treatment. For prevention avoid rat bite. The disease is amenable to salvarsan. Penicillin is reported to be effective.

CHAPTER XXII

PROTEUS AND PSEUDOMONAS

The *Proteus* organisms are characterised by a high degree of pleomorphism, hence the name. They also possess very active proteolytic properties and exhibit the tendency to spread rapidly on moist solid media. They are actively motile with peritrichate flagella. Lactose is not fermented, but many other sugars, such as glucose and saccharose, are fermented with gas production. The discovery of certain types, designated X strains, is of great interest because of their serological affinity with *Ricketsia*. About eight species on the whole have been described. *Proteus vulgaris* is the type species. Morgan's bacillus (No. 1) is included in this genus.

These organisms are usually found in putrefying animal and vegetable matter, faeces, sewage and soil. Their frequent presence in the infected wounds is thus easily explainable.

Morphology and Staining. These bacilli are highly pleomorphic, straight or curved rods, 1·0–3·0 by 0·5–1·0 micron, with rounded ends; arranged singly, in pairs and frequently in long chains; actively motile with peritrichate flagella; not sporing, usually not capsulated; Gram-negative.

Growth Requirements. They are aerobic and also possess the faculty of growing in the absence of oxygen. The optimum temperature is low, 25° C., but they grow well at 37° C. Their nutritional requirements are simple and the bacilli grow luxuriantly on all laboratory media.

Cultural Characteristics. On solid media, like agar, a spreading type of growth is obtained. The phenomenon is referred to as " swarming ". It is more pronounced if the medium is moist; the rapidly spreading sheet of growth is very thin and can scarcely be made out from the medium. It is probably due to the active motility of the organism. Owing to the swarming nature of *Proteus*, the isolation of other organisms from a mixture containing it is unusually difficult. On blood agar the beta type

of haemolysis occurs. Moderate growth occurs in broth with slight turbidity. Cultures emit a putrefactive odor.

Biochemical Reactions. Lactose and mannite are never fermented; glucose is always fermented with the production of acid and gas. Action on saccharose, maltose and salicin is variable. Most strains produce indole. The action on maltose and saccharose and the production of indole are useful in the classification of the several members of the genus. Litmus milk is rendered alkaline after a slight initial acidity and is usually peptonised slowly with or without preliminary clotting. The methyl red test is usually positive and the Voges-Proskauer reaction negative. Nitrates are reduced; hydrogen peroxide is formed. *Proteus* exhibits marked proteolytic properties; gelatin is rapidly liquefied and urea is decomposed with the formation of ammonia. A haemolysin is formed, acting on rabbit's blood.

Resistance. They are equally sensitive to bactericidal agents as other vegetative bacteria . Cultures remain viable over long periods. The group is insensitive to penicillin.

Antigenic Structure. *Proteus* is a serologically heterogeneous group. In the motile state, all species contain both H and O antigens, but the non-motile strains have, of course, only the O antigen.

Type X 19 and to a less degree type X 2 are agglutinated by sera from typhus patients, the former in very high dilutions (up to 1 : 50,000). Their original isolation from the urine of typhus patients may have been only fortuitous. Though these organisms are not causally related to typhus fever, the test is quite reliable and extensively used for the diagnosis of this disease (see under *Rickettsia*). It is called Weil-Felix reaction. This apparently non-specific reaction is due to the presence of a common antigenic fraction in these proteus bacilli and the pathogenic rickettsia; the common moiety is probably polysaccharide in nature. The X 19 and X 2 strains ferment saccharose, maltose and salicin, producing acid and gas; indole is formed. They are serologically distinct from other members of the group.

Another type, the Kingsbury strain or XK, is likewise agglutinated by the sera of patients suffering from tsutsugamushi or the mite typus (see Chapter XXXIV) and is used for the diagnosis of this condition. It ferments saccharose, but not maltose and salicin. It does not form indole.

Pathogenicity and Toxin Production. No soluble toxin is elaborated by any member of this genus.

Proteus possesses feeble pathogenic properties. Alone or in association with other pathogens, it is found in otitis media, cystitis, pyelitis and other suppurative and septic lesions. It is often a cause of intractable cystitis. It has been incriminated as one of the organisms causing infantile diarrhoea and food poisoning. *Proteus* is of variable virulence to laboratory animals. Some strains are very virulent and cause on intraperitoneal inoculation death in a few hours, presumably from toxaemia.

Diagnosis. The organism is isolated and identified by its general properties. The presence of it in materials causes considerable trouble in the isolation of pyogenic cocci from them (p. 387).

Treatment. A heat-killed vaccine is found useful in chronic conditions due to *Proteus*.

Proteus Morgani. This bacillus was first isolated in 1906 by Morgan from cases of summer diarrhoea. It resembles the proteus organism generally but differs in its proteolytic inactivity. Growth on agar produces bluish white or greyish glistening colonies. Swarming occurs at a lower temperature, between 20°–28° C., on 1 per cent. agar (see above). It has only very restricted activity on carbohydrates; it produces acid and a small amount of gas in glucose and rarely acid in xylose; other sugars are not attacked. Delayed fermentation of saccharose has been reported. Litmus milk remains neutral or becomes alkaline but not digested. Indole is formed. Gelatin is not liquefied.

Morgan's bacillus is one of the organisms incriminated as the cause of summer diarrhoea. Occasionally it is found in the stools of dysentery cases. The isolation of it from cases of paratyphoid-like fevers has been reported. It may also be present in normal faeces. In mice it causes a spontaneous epidemic of enteritis.

PSEUDOMONAS

The chief generic characteristics are: short, straight rods, usually motile by means of polar flagella; non-sporing, non-capsulated, usually Gram-negative; inactive on carbohydrates; aerobic and facultatively anaerobic; frequently elaborating a

water soluble blue, green or yellow pigment which diffuses out of the bacterial cell. They are widely distributed in nature, mostly in water and soil. About thirty species have been so far described. Two species are of some medical interest—*Pseudomonas pyocyanea* and *Pseudomonas fluorescens*. Some species are pathogenic to chicken, reptiles and caterpillars and others to plants.

Pseudomonas Pyocyanea. *Ps. pyocyanea* is commonly called the organism of blue pus.

Ecology. This organism is a natural denizen of the intestinal canal of man and animals, water and sewage; it is also found in open wounds and sinuses, mixed with other pathogens.

Morphology and Staining. They are straight rods, 1·5–3·0 μ by 0·5 μ with parallel sides and rounded ends, arranged singly, in pairs and short chains; actively motile, possessing one to three flagella at one pole, develop neither capsule nor spores. They are easily stained but lose primary stain in the Gram's method.

Growth Requirements. The organism is an aerobe but can also grow anaerobically. Growth occurs between 12° and 43° C.; the optimum temperature is 30°–37° C.

Cultural Characters. *Ps. pyocyanea* grows readily on all media. On agar colonies are large, low convex, smooth, spreading, greyish with opaque centre and translucent periphery; margin entire or irregular; butyrous and easily emulsifiable. The medium is tinged greenish blue. In broth there is luxuriant growth with dense turbidity, thin white surface growth and slight deposit; the medium is coloured greenish blue, later turning yellowish green and in old cultures dark green.

Biochemical Activity. It is practically inert on carbohydrates, glucose and galactose being the only ones to be fermented with the production of acid, but not gas. Litmus milk is digested; indole is not produced. Gelatin is rapidly liquefied and hydrogen sulphide is produced. *Ps. pyocyanea* manufactures two types of pigments: *pyocyanin* and *fluorescin*; the former, greenish blue, is soluble in water and chloroform and the latter, fluorescent and yellowish green, is soluble in water but not in chloroform. They do not colour their colonies but colour the substrate. They are best developed at room temperature and the best medium is peptone water. Pyocyanin is elaborated only by *Ps. pyocyanea*, but fluorescin is formed by many other species of *Pseudomonas*,

including *Ps. fluorescens.* Pyocyanin is the first instance found of a phenazine derivative occurring naturally. The organism produces an antibiotic substance, called *pyocyanase*, which is lytic to many other bacteria, such as the anthrax bacillus, staphylococcus and streptococcus; this substance is lipoidal in nature. It would appear that pyocyanin also possesses antibiotic properties. *Ps. pyocyanea* also synthesises a haemolysin.

Resistance. Heat at 55° C. destroys it in one hour. It is insensitive to penicillin. Cultures will keep alive for several months at the laboratory temperature.

Pathogenicity and Toxin Production. No exotoxin is produced. The organism is only feebly pathogenic. It is constantly found in open wounds and discharging sinuses and it is probable that its role in most of these is only that of a secondary invader. *Ps. pyocyanea* may also cause primary infection, particularly in persons with impaired vitality and in young subjects. It may cause a highly fatal form of gastro-enteritis in infants and children. Rarely it may invade the blood stream and a fatal form of septicaemia has been reported by several observers. It is frequently found in suppurative conditions, like peritonitis, mastoiditis, sinusitis and in suppurative lesions of the urinary tract; in these it is sometimes the sole organism present, but at other times it is associated with other pyogenic bacteria. A chronic form of otitis media and externa is almost always associated with it and is very intractable.

The guinea-pig is very susceptible and about quarter of a cubic centimetre of culture injected intraperitoneally kills it in twenty-four hours. Subcutaneous inoculation with dead cultures has been found to incite the production of agglutinins. Rabbits are less susceptible than guinea-pigs; in both local abscesses are produced by the subcutaneous inoculation of small doses of live culture.

Diagnosis. Diagnosis is very easy; the mere cultivation will reveal the presence of the organism due to the development of the characteristic colour. But the fixation of its pathogenic role is exceedingly difficult and should not be undertaken light-heartedly.

Ps. fluorescens is a saprophyte commonly present in water and soil. It produces only fluorescin. Though rarely, it has been encountered in certain chronic suppurative conditions like otitis media.

CHAPTER XXIII

MISCELLANEOUS BACTERIA

LACTOBACILLUS

The genus *Lactobacillus* consists of rod-shaped cells, often long and slender and sometimes pleomorphic, non-motile, non-sporing and Gram-negative. They are microaerophilics, facultative anaerobes or strict anaerobes. They ferment carbohydrates, producing, as a rule, lactic acid. Little or no proteolytic activity is associated with them. They are widely distributed in nature and found in vegetable and animal products. About forty species have been described, but none of them is pathogenic to man. Their main interest is in their use in industrial fermentation processes. No satisfactory classification of these organisms has been arrived at.

Members of this genus have been described from such widely different situations as fermenting milk—*L. caucasicus* and *L. bulgaricus*, normal human vagina—Doderlein's bacillus which is now accepted as *L. acidophilus*, stomach contents in gastric carcinoma—Oppler-Boas bacillus, faeces of breast-fed infants—*L. acidophilus* and *L. bifidus*, faeces of mammals, fishes and invertebrate animals, normal mouth, stomach and intestines of man, carious teeth—*L. odontolyticus*, silage, soil and sewage.

Morphology and Staining. They are generally large organisms arranged singly, in end to end pairs and in chains. Pleomorphic forms, clubbed, knobbed, curled, spiral, vacuolated, filamentous, etc., are common, especially in old cultures. In some species Y-shaped forms, giving an appearance of branching, are very common. The *Lactobacilli* develop neither flagella nor spores and are Gram-positive.

Growth Characters. They are microaerophilic, facultatively anaerobic or strictly anaerobic; after a few subcultures, they may grow aerobically. Generally, they grow between 25° and 46° C. with an optimum of 37°–40° C. The optimum reaction seems to be pH 6·0 with a range of pH 5·4–6·8. Pantothenic acid, riboflavin and pyridoxin appear to be essential for growth.

Cultural Characters. They do not grow well on ordinary media. The addition of whey, milk, blood or glucose improves growth. But it is never profuse. *Lactobacillus* gives rise to two types of surface colonies: minute, round, opaque, whitish and surrounded by a zone of turbid agar; or minute, irregularly round, greyish, transluscent and with no zone of turbidity.

Biochemistry. Varying number of sugars are fermented by different strains. Practically all strains ferment glucose and lactose. Indole is not produced. Most species produce acid in milk, forming loose clot which does not shrink and express whey; the litmus is frequently decolorised. As a rule, it is lactic acid that is produced, which may be up to 2·7–3·7 per cent.

Resistance. They are not particularly resistant to the common bactericidal agents. Heat at 60° C. destroys them in thirty minutes. Unlike other non-sporing bacteria, they are very resistant to acid, hence the names " aciduric " and " acidophilic". This property is made use of in their isolation. The material is incubated in 0·5 per cent. acetic acid broth for 1–3 days and then subcultured on 2 per cent. glucose agar.

No pathogenicity has been attributed to these organisms. Those found in the carious teeth are mere saprophytes. *Lactobacilli* are normally found in the mouth and intestines, more abundantly in vegetarians.

The administration of living culture of *L. acidophilus* and *L. bulgaricus* is employed as a therapeutic measure by some workers, especially in America, in certain intestinal disorders. They believe that it will suppress the growth of putrefying intestinal bacteria, relieve constipation and improve the so-called auto-intoxication of intestinal origin. But the results so far achieved have not fulfilled the hope.

FUSOBACTERIUM

Fusobacterium is the generic name given to a group of organisms which exhibit the following characters: obligate parasites; anaerobic or microaerophilic; cells frequently elongated and fusiform, staining somewhat unevenly; filaments sometimes formed; non-branching; non-motile; non-sporing; reaction to Gram variable; growth in laboratory media feeble. Several species have been described.

Fusobacterium Plauti-Vincenti. This organism is found in various ulcerative and necrotic lesions of the mouth, throat, intestines and genitalia. It is frequently associated with *T. vincenti.* It is constantly present in the chronic condition called tropical ulcer. It has probably some causal relationship with Vincent's angina. Its exact pathogenic role has not been determined. It does not appear to be pathogenic to guinea-pigs and monkeys.

It is 0·5–1·0 by 8–16 microns in size, occurring singly or in pairs. Pleomorphism is common, especially in culture. It is straight or curved, thickened in the middle and tapering to the ends. It is non-motile, Gram-negative and granular in appearance. Dilute carbol fuschin stains it well.

The fusiform bacillus is an anaerobe. The optimum temperature is 35°–37° C. Growth is slow. On ordinary media growth is poor to nil. The addition of serum or blood renders them suitable. A medium containing one part of blood to three of agar has been found to be satisfactory. In this it grows in small colonies resembling those of streptococci. Often there is a zone of green haemolysis about the colonies.

Glucose, levulose, saccharose, maltose and sometimes lactose are fermented without gas production; inulin or mannite is not attacked. Indole is not formed.

BACTEROIDES

The scope of this genus is still unsettled. Many apparently heterogeneous species are now included in it and this arrangement should be considered as tentative. Bergey defines the genus as: motile or non-motile rods without endospores; obligate parasites; may or may not require enriched culture media; Gram-negative.

Bacteroides Fragilis. The cells of this species are small slender rods with rounded ends, occurring singly or in pairs. They are Gram-negative and non-motile. Some authorities include this organism in the genus *Fusobacterium.*

It is an obligate anaerobe and grows best at 37° C. It will grow on the usual laboratory media, but the growth is scanty. The colonies are small, grey and irregular. A variety of sugars, such as glucose, maltose, saccharose and arabinose, are fermented without gas production.

Bacteroides fragilis has been isolated from cases of appendicitis, pulmonary and hepatic abscesses, septicaemic conditions

associated with metastatic abscesses and infections of the urinary tract. Its exact pathogenic role in these conditions has yet to be determined. On subcutaneous inoculation, some strains produce abscess in rabbits, guinea-pigs and mice.

Bacteroides Melaninogenicus. This species consists of small Gram-negative, non-motile rods, usually arranged in pairs. It is an anaerobe and grows best at 37° C. On blood agar it grows in black colonies due to the formation of a melanin-like substance. Better growth is obtained in mixed culture than when the organism is in the pure state. It attacks glucose, lactose, maltose, mannite, saccharose and a few other sugars, producing only acid. It is markedly proteolytic on native proteins. It is naturally present in the oral cavity and external genitalia of man. Its presence has been noted in infected wounds, focal infections of kidneys, in faeces from chronic amoebic dysentery and in peurperal infection. Whether it has any pathogenic role, is yet undetermined. It is not pathogenic for rabbits, guinea-pigs and mice.

Bacteroides Ramosus (Fusiformis ramosus). This is another species of undetermined systematic position. The cells comprising it are tiny slender rods which often show branching Y forms and V forms. The organism is Gram-positive, not motile, and not sporing. The cells are arranged in pairs or short chains. *Bacteroides ramosus* is an obligate anaerobe. It grows on ordinary media but slowly. It is frequently encountered in appendicitis and in gangrene of the lungs. Its occurrence in gas gangrene and often in the blood has been recently reported. It is pathogenic to experimental animals, causing abscess on subcutaneous injection into rabbits and guinea-pigs and toxaemic death of these animals on intravenous administration.

Actinomyces Necrophorus. This is yet another species of unsettled taxonomic position. Some authorities group it with *Fusobacterium.* The cells are rod-shaped with 0·5–1·5 microns wide, forming long filaments up to 80–100 microns long, occasionally showing branching. The short forms are motile. The organism is Gram-negative and beaded appearance is common. It is a strict anaerobe and its optimum temperature is 37° C. The colonies on agar are small, dirty white, circular, opaque discs. Glucose, maltose and levulose are fermented, with the production of but acid. Indole is produced. Gelatin is not liquefied.

The necrophorus bacillus produces an exotoxin. It causes diphtheritic and necrotic lesions, "necrobacillosis," in many animals—horses, mules, sheep, pigs, cattle and others. In these multiple abscesses are formed in the internal organs and the organisms are found in large numbers in these lesions. Extensive gangrenous reaction and sloughing of the tissue may occur. A similar organism has been seen in suppurative lesions, such as hepatic and pulmonary abscesses and ulcerative colitis, and even in the blood in man. The organism is also present in the normal alimentary and genital tracts of man. It is highly pathogenic to experimental animals, some strains producing on subcutaneous inoculation spreading necrotic lesions in rabbits which end fatally.

LISTERELLA

The genus *Listerella* contains only one species, *L. monocytogenes*. This microbe was first isolated from rabbits suffering from a disease characterised by a large mononuclear leucocytosis and named at that time *Bacterium monocytogenes*. Its correct systematic position is still doubtful.

Listerella monocytogenes is a small Gram-positive bacillus, $1 \cdot 0–2 \cdot 0 \mu \times 0 \cdot 5 \mu$, feebly motile by means of a single very long polar flagellum. It is non-capsulated and non-sporing. The cells are arranged singly, in V-shaped or parallel pairs or in short chains. Often a beaded appearance is seen.

The organism is aerobic and facultatively anaerobic. The optimum temperature is 37° C. It grows best on media enriched with liver extract or blood. The colonies on solid media are small, transparent by transmitted light and milk white by reflected light. Beta haemolysis is produced on blood agar.

Its biochemical activity is restricted. Glucose, rhamnose and salicin are fermented without the production of gas. Maltose and saccharose are fermented irregularly and slowly. Indole is not formed and gelatin not liquefied.

A few cases of *Listerella* infection in man, meningitis and septicaemia, have been reported. The infection is probably acquired from infected animals. Natural infection of animals is not infrequent and it is characterised by monocytosis. Swine, sheep, fowls, and rodents, like mice, guinea-pigs and rabbits, may all be affected.

DIALISTER

The generic characters are: minute rod-shaped cells, occurring singly, in pairs and short chains; non-motile; anaerobic; grows only in media containing fresh, sterile tissue or ascitic fluid; strict parasites.

Dialister Pneumosintes. The cells are very minute, coccobacillary, measuring 0·15–0·3 micron long and occurring singly, in pairs, short chains and masses. They pass through Berkefeld V and N filters. *Dialister pneumosintes* is non-motile and Gram-negative. It stains a deep purple with polychrome methylene blue. It is a strict anaerobe. The optimum temperature is 37° C. The organism is fastidious in its nutritional requirements, growing well in Smith-Noguchi medium or in glucose agar containing rabbit blood and incubated anaerobically. Of the ordinary sugars, it ferments only glucose, producing acid. It is normally found in the nasopharynx of man. It was first isolated from the nasopharynx of cases of epidemic influenza in the first thirty-six hours of the disease and was for sometime believed to be its cause. It is apparently non-pathogenic.

THE PLEUROPNEUMONIA GROUP OF ORGANISMS

This group contains very minute, extremely pleomorphic organisms showing granules, rings, coccoid forms, filamentous and other aberrant forms. Some forms can pass coarse bacterial filters. The smallest size noted is 125 to 250 millimicrons. These organisms are non-motile and stain poorly with ordinary dyes but well with Giemsa. They are Gram-negative. They are facultative anaerobes. They can grow in artificial media and independently of living tissue. Both saprophytic and parasitic forms exist. Many species are pathogenic to animals, the most important being the organism of bovine pleuropneumonia. The exact relationship of these organisms among themselves and with other groups of organisms is not known. Owing to their filterability some authorities group them with the viruses and others with the ricketssiae because of their tendency for an intracellular existence in the host tissues. Infection with these organisms produces immunity but not so solid as that produced by some of the viruses.

CHAPTER XXIV

PASTEURELLA

Pasteurella is the generic name for a group of small, Gram-negative, ovoid to elongated, bipolar staining bacilli, possessing limited biochemical activity. They are parasitic on man, animals and birds.

The genus includes *Pasteurella pestis* or the plague bacillus, *Pasteurella septica* or the organism of haemorrhagic septicaemia of animals and birds and *Pasteurella pseudotuberculosis* which causes a tuberculosis-like disease in certain rodents. Following Bergey, the organism of tularaemia, *Pasteurella tularensis*, is included in this genus. Many members of this genus cause in various animals and birds the disease known as haemorrhagic septicaemia. Organisms isolated from different animal hosts have been assigned by some authorities separate specific names derived from the animal species mainly affected. Thus, we have *Past. aviseptica* of birds, *Past. muriseptica*, a natural pathogen of wild rats, *Past. lepiseptica* causing a natural disease in rabbits, *Past. suiseptica* of swine plague. *Past. boviseptica* which causes a haemorrhagic septicaemic disease in cattle, horses and deer, and others. But the differences between these are, as known at present, too insufficient to justify the creation of new species. Even the host adaptation is not always exclusive but loose. Hence, these organisms are better treated as belonging to the same species, *Past. septica*, pending clarification of their interrelationship by further studies.

Pasteurella Pestis (L., pestis, plague). The plague bacillus was discovered and described independently by Kitasato and by Yersin in 1894 during an epidemic at Hongkong. In the years following, plague was established to be a disease primarily of rodents by several workers. Its transmission to man through the agency of infected fleas, although reported by many workers previously, was conclusively proved by the British Plague Commission in India in 1906.

Ecology. *Past. pestis* is a strict parasite of rodents and man. It is also found in the infected flea. The organism is found in large numbers in the buboes, blood, pleural effusion, spleen and liver of infected rodents and man and also in the sputum in pneumonic plague.

Morphology and Staining. They are small, plump, ovoid rods, $1 \cdot 5 \times 0 \cdot 6 \mu$, with rounded ends and convex sides and occurring singly. The tendency to chain formation is observed in fluid culture. *Past. pestis* shows a high degree of pleomorphism, particularly in tissues. Involution forms also constitute a conspicuous feature, especially in old cultures. Such forms are easily developed in the presence of 3 per cent. sodium chloride. Coccoid, snake-like, yeast-like, filamentous forms and shadow forms may all make their appearance in twenty-four hours. The plague bacillus is non-motile, non-sporing and capsulated in tisssues. In cultures grown at 37° C. it develops a slimy gelatinous envelope.

The organism stains easily. It is Gram-negative and not acid-fast. The tendency to bipolar staining, more marked in tissue forms, is a characteristic feature of the plague bacillus. It is brought out better when stained with a weak stain, like methylene blue. This staining peculiarity is due to the more intense staining of the polar areas than the intervening portion of the bacillary body and not due to the presence of metachromatic granules.

Growth Requirements. This microbe is aerobic and facultatively anaerobic. The optimum temperature of the pestis bacillus, unlike other human pathogens, is 25°–30° C. The temperature range is between –2° and 45° C. (Sokhey). The optimum pH is 6·2–7·0. The plague bacillus grows readily on all media and has no special nutritional needs. When grown on simple media, some strains may require nicotinamide or thiamine or both.

Cultural Characters. The plague bacillus grows on all media but only slowly and sparsely. On agar the colonies are small, round, greyish white, glistening, translucent and finely granular discs, butyrous in consistence and easily emulsifiable. Older colonies show a raised centre and flat periphery. There is moderate growth in broth, which may be turbid or clear with flocculent or powdery deposit at the sides or bottom of the tube. Older cultures show pellicle with streamers into the fluid (stalactite). Such streamers can be made to develop in young cultures by

floating sterile oil on the surface and incubating them undisturbed for a few days. Though it is highly characteristic of *Past. pestis*, it is not peculiar to it.

Biochemical Activity. *Past. pestis* produces acid without gas in glucose, mannite, maltose and a few others. Lactose is not attacked. It does not produce indole. Nitrates are reduced to nitrites. Milk is rendered slightly acid but not clotted. Broth is turned alkaline. Bile salts do not inhibit growth. No haemolysin is produced nor gelatin liquefied.

Resistance. Its resistance to injurious agents is not high. Heat at 55° C. kills the organism in five minutes and phenol in 0·5 per cent. concentration in fifteen minutes. Cultures kept at low temperature may survive long. Drying is rapidly lethal; so also exposure to sunlight.

Antigenic Structure. *Past. pestis* is a serologically homogeneous group. It appears to have a heat labile envelop antigen and a heat stable somatic antigen. The latter is common to *Past. pseudotuberculosis* and cross agglutination between these two organisms is a marked feature.

Toxin Production. No soluble toxin is produced by *Past. pestis*. The endotoxin is the nocuous agent. Virulence and immunising properties most probably also depend upon the presence or absence of the envelop antigen. This antigen develops only at 37° C. and is destroyed at 100° C.

Pathogenicity. *Past. pestis* causes disease in man and certain rodents, like rats, ground squirrels and others. Domestic animals are relatively immune. With the exception of sparrows, birds are quite resistant. The organism is infectious for mice, guinea-pigs, rabbits and monkeys. Subcutaneous inoculation with a fresh culture kills the mouse or guinea-pig in two to five days. Infection can also be induced by the cutaneous route by rubbing the infected material on the shaven skin. The same thing can be done through the nasal or conjunctival mucosa. This procedure is useful when the infected material contains extraneous organisms.

The post-mortem appearance in guinea-pigs is characteristic. The tissue at the site of inoculation is necrotic with haemorrhagic oedema all round. The regional glands are enlarged, soft and necrotic with periglandular inflammatory and haemorrhagic oedema. There are subcutaneous and subserous haemorrhages.

The spleen is enlarged congested and studded with miliary, soft, grey nodules. The liver is enlarged and peppered as it were with tiny necrotic foci. There is effusion into the pleural cavity. The causal germ is found in large numbers in all the organs, especially numerous in the necrotic areas. It should be remembered that a condition closely simulating this is given rise to in pseudotuberculosis of guinea-pigs caused by the allied organism *Past. pseudotuberculosis.*

Epidemiology. Plague was known from very ancient times as one of the greatest scourges of the human race. In relentless waves it used to sweep over the world with frightful mortality. It has been estimated that the " Black Death " of the 14th century killed over 25 million people in Europe, nearly a quarter of the then population. About 70,000 people perished in London alone during a severe outbreak in 1665—the Black Death of London. Plague was comparatively quiescent in the 18th and 19th centuries, being then confined to the endemic areas. A fresh wave started in 1894. Beginning in the Yunan province of China, it soon reached Hongkong and thence spread practically to all countries of the world. Through merchandise Bombay was affected in 1896. During this pandemic India suffered terribly. Her recorded death rate (for British India) from plague for twenty years beginning from 1898 was 10¼ million.

The present endemic foci in the world are: India, Burma, Ceylon, Iraq, portions of S. America and Africa, S. E. Russia, Siberia, Manchuria, China, the East Indies, West Indies and California.. In this country the Punjab, Bombay, the United Provinces, Hyderabad and Madras are the chief endemic centres, Tinnevelly, Salem and Bellary being the chief focal points in the last.

Plague occurs more in the warmer regions. In the tropics the greater incidence is in the cold weather, January to April or May, as a temperature over 80° F. associated with low humidity is inimical to flea life. Besides, rats during the hot season go far afield on their forage expeditions, thus reducing the opportunity for their close contact with man.

Plague is a disease primarily of rodents. From them it spreads to man through the agency of infected fleas. Rats form the main victims of plague. It is so in India. Other reservoir hosts are: the ground squirrel in California, the susliks in S. E. Russia,

the tarabajan in Manchuria and the gerbillus and the multimammate mouse in Africa, all rodents. There is reason to believe that plague infection among rodents is extending, which constitutes a greater potential danger. Among the rats, the small black house rat, *Rattus rattus*, and the large grey sewer rat, *Rattus norvegicus*, are most affected, the latter more commonly than the former. The infestation rate of fleas in *R. norvegicus* is double that in *R. rattus*. But due to its closer relationship to man, the small black house rat is more dangerous.

In the endemic areas the disease exists in rats and other rodents in a chronic and inapparent form. The carrier animals may be apparently healthy, but the infection lurks in them in the form of large abscesses in the liver or spleen. Sporadic cases may also occur in the rodents as well as in man. Such inapparent and sporadic forms serve as the connecting link between epizootics. When conditions become favourable, the dormant lesion in the *R. norvegicus* flares up, forming the first case to start the epizootic. The acute disease in the rodents is essentially septicaemic and the blood-sucking flea host is sure to be spontaneously infected. Fleas may, therefore, indirectly spread the infection among rats. As *R. norvegicus* is a ferocious fighter, among them the infection is also spread directly through bite. Ten to fifteen days after the epizootic starts among the grey rat, it is transmitted to the house rat, among which the disease soon spreads as a mass infection. This is followed after a similar interval by the outbreak of epidemic among man. Thus, epidemics are always heralded by epizootics among rats or other rodents and rat falls are, therefore, rightly taken as the harbingers of human plague. When a rat dies of plague, the fleas leave it in search of new hosts. Usually the flea finds another rat, but should it happen to come across man, the insect infects him by virtue of its previous infectious feed on the deserted rat. That is how man happens to be the victim and the infection transmitted to him.

The rat fleas responsible for transmission in this country are *Xenopsylla cheopis* and *Xenopsylla astia*, the former being far more important than the latter. In certain regions *Ceratophyllus fasciatus* acts as the insect vector. The plague bacilli live and multiply in the intestine of fleas. How long they do so and how long fleas remain infective, depend upon several factors. During the epidemic season it is about fifteen days and during the off

season seven days. In the laboratory a flea can be kept infected for about 47 days. The clearance is probably due to the insect developing immunity.

The mechanism of infection now accepted is somewhat as follows: the blood that is sucked from the rat contains plague bacilli. It clots in the proventriculus of the flea and in the clot the microbe multiplies rapidly. The clot mechanically blocks the proventriculus, preventing another feed. The insect becomes thirsty and hungry and tries to suck blood from the new host. In this act the insect makes tremendous efforts, during which probably a minute fragment from the infected clot becomes detached. This is regurgitated and implanted in the new host through the bite puncture. Another possible mode of transmission is through infected faeces. The organism is undoubtedly present in the excreta. Through the bite wound the infected faeces or blood of the flea, crushed by scratching as the result of irritation caused by bite, is rubbed in.

Pneumonic plague is highly contageous. Its incidence is independent of rodents or fleas. It is characterised by peribronchial inflammation soon followed by haemorrhagic pneumonia of a lobular or lobar type. The organism is found in abundance in the sputum and the spread is directly from man to man through infected droplets. The pneumonic type may originally be starting from plague pneumonia. Plague seems to take a pneumonic turn in the ground squirrel and tarabajan, and a direct spread of pneumonic plague from these to man is not out of the picture of the epidemiology of pneumonic plague.

Rat being the reservoir host, the disease always starts in the neighbourhood of buildings, particularly grain store, warehouses, godowns and shops. From such localities it spreads to adjoining or distant places. The spread to distant places is usually by way of the trade and travel routes and by transport agencies, like trains and ships, harbouring infected rats. The same is also served by merchandise, such as bales of cotton and bags of grains, carrying infected rodents and fleas. A human case coming to a locality cannot start an epidemic as the vector flea, not being naturally parasitic on man, is not infected by him. The rodents and their fleas have to get infected first before man can get the infection. That is, the reverse condition of transmission of infection from man to fleas and from fleas to rats does not occur as the flea is not a parasite of man.

The immunity mechanism against plague would appear to be very inefficient. Also, the organism is very invasive and overpowers the defence forces of the body before any resistance can be put up. Apparently, all races, all ages and both sexes are extremely susceptible. Those who escape from getting the disease during epidemics do so because they escape from bite of the infected flea and may not be due to any inherent resistance. The incubation period of bubonic plague is 5–7 days and of the pneumonic type 1–3 days. The case mortality in the former is 60–90 per cent. and in the latter 100 per cent. It is almost 100 per cent. in the septicaemic form but very low in the cutaneous type.

Immunity. Antibodies, like agglutinins, lysins and opsonins, are formed during infection, but how far they are protective, is not clear. At any rate, their formation is too late to be effective in the large majority of cases. Recovery is usually followed by lasting immunity.

A certain amount of protection may be conferred on experimental animals by active immunisation. An antiserum has been prepared in the horse, but its therapeutic value is doubtful.

Diagnosis. It is necessary to be familiar with the methods of diagnosis of plague both in man as well as in rats or other rodents. The diagnosis of plague in man rests on the finding of the organism in the materials obtained from the patient, its isolation and identification. Serology affords but little aid. The juice aspirated from the bubo, blood from the late bubonic and from the septicaemic types and the sputum in the pneumonic form will all contain the organism in large numbers. In about 5 per cent. of the bubonic type vesicles are developed at the site of bite. Fluid from such vesicles also contains the bacilli. Hence, examination of stained smears of these materials will show large number of bacilli with the characteristic bipolar staining and marked pleomorphism. Cultures with these materials are then made and the isolation of the organism proceeded with. The biochemical activity of the isolated organism is studied and its identity established by doing an agglutination test with the specific serum.

Confirmation may also be sought by animal inoculation, employing a white mouse or guinea-pig. The material is directly inoculated into the animal subcutaneously or intraperitoneally. But, if it is contaminated with other organisms, as it happens when it is taken from a bubo or spleen after death, it is rubbed

over the shaven skin. The plague bacilli penetrate the skin but the contaminants do not. The animal dies in two to five days with characteristic post-mortem findings.

Serology is of little practical value in diagnosis. The patient's serum may give a positive agglutination reaction with the specific antigen in a titre up to 1 : 100.

Pseudotuberculosis of guinea-pigs and rats gives a somewhat identical picture. But it can be differentiated by the fact that the causative organism, *Past. pseudotuberculosis*, is motile at 22° C., while the plague bacillus is non-motile at all temperatures and that the former is non-pathogenic to white rats. Tularaemia may rarely cause confusion, when isolation of the organism and further study will clinch the diagnosis.

Diagnosis of Infected Rats or Other Rodents. This may be often required as rat falls are harbingers of human plague and a correct diagnosis made betimes may enable proper prophylactic measures to be taken before cases begin to appear in man. The post-mortem appearances in rats are characteristic: pink paws; single cervical bubo; periglandular inflammation and oedema of neck; subcutaneous congestion and haemorrhage, especially of the submaxillary region; haemorrhages into the lungs, pleura, adrenals, kidneys and other organs, and pleural effusion. Microscopic examination reveals large number of organisms in the tissues, especially in the spleen and bubo. Confirmation should be obtained by cultural, serological and animal inoculation tests as described above.

Thermoprecipitin Test. This test is particularly useful if the tissue has undergone putrefaction. One gram of the infected tissue is macerated in 5 ml. of distilled water and boiled for 5 minutes. It is cooled and filtered. A few drops of the specific serum are then run on to the surface of the filtrate contained in a narrow test tube. Precipitation occurs at the interface in about 5 minutes in positive cases. The phenomenon is hastened at 37° C.

Treatment and Prophylaxis. A specific serum has been used in the treatment of human cases. It is of doubtful value. Even so it has to be employed, for nothing should be left undone in such a fatal infection with no other remedy offering any hope. It should be given as early in the disease as possible, in 80–100 c.c. doses, always intravenously and repeated every six hours until

recovery takes place. It may also be given to those who are proceeding for plague work.

Investigations conducted by the Haffkine Institute, Bombay, tend to show that the sulphonamide compounds are valuable in the treatment of bubonic plague.

Prophylaxis has been aimed at breaking the rat-flea-man chain. The destruction of rats has been tried with but little success, as they multiply with enormous rapidity. A more hopeful measure is to render all buildings in the endemic area, such as dwelling houses, godowns, shops and granaries, unsuitable as rat habitat. Similarly, all merchant vessels touching ports in or near the endemic area should have rat-proof arrangements. When coming from any infected port, ships should be put under strict quarantine and all protective measures taken before allowing them to enter the port.

Fleas thrive where filth and shelter are easily available. Hence, household cleanliness is important as a prophylactic measure. Cases of bubonic plague may be treated in a general ward provided blood sucking vermins, including fleas and bugs, are carefully excluded from all beddings and cots. Those who attend on cases of pneumonic plague should protect themselves against droplet infection.

General preventive measures, such as evacuation of dwelling houses, isolation of the sick, removal of overcrowding and attention to personal hygiene, should all be taken. The isolation of the sick, however, is only of restricted value in controlling the spread of bubonic plague, as man is not directly infectious to man and as fleas do not generally infest him. But isolation is of supreme importance in the pneumonic type.

The protection of the individual by active immunisation is of value. In the Haffkine's method of preparing vaccine, suitable strains are grown in digest broth for four weeks at 27° C. and killed by heat at 55° C. for fifteen minutes. To this is added 0·5 per cent. phenol as a preservative. The vaccine is tested for sterility and standardised by the mouse protection test. The dosage for adult is 4 c.c., given in a single dose subcutaneously. The dose for children is less, proportionately to their age. A certain degree of immunity is developed which lasts for some months to a year. In varying degrees it wards off the disease or modifies it in favour of the individual. Besides the general

population exposed to it, everybody who is concerned with plague work should be immunised every year.

Vaccine prepared in this way has been criticised. If the envelope antigen is the effective antigen and if it develops best at 37° C., the more rational method is to grow cultures for preparing vaccine at 37° C. Such a vaccine has, indeed, been prepared and greater success claimed by those who have employed it. A live attenuated culture has also been used for vaccination in certain areas, claiming still greater success rate. A live culture, however, may not be without potential danger.

Pasteurella Tularensis. *Pasteurella tularensis* is included by some authorities in the *Brucella* genus with the members of which it is serologically related. It is a small organism, 0·3–0·7 μ long. It is capsulated in tissues and does not develop spores; it is non-motile. The organism is somewhat difficult to stain. It is Gram-negative and bipolar staining may be evident. It causes tularaemia.

It is a strict parasite, occurring in large numbers in the lesions and blood of naturally infected animals and man. Recently, *Past. tularensis* has been reported to be present in streams, thus probably accounting for the epizootics occasionally observed among beavers.

Growth Requirements. It is aerobic and facultatively anaerobic. Its optimum temperature is 37° C. It does not grow on ordinary media and cultivation is difficult. Special media, containing egg yolk, cystine, glucose, blood and agar, are necessary for its growth. The colonies on solid media are minute, transparent and drop-like. It has been shown recently that the organism is capable of growing in liquid media.

It ferments a number of sugars, including glucose, maltose and mannite. But this is of no differential value.

Tularaemia is a natural plague-like disease occurring in wild rodents, such as rabbits, hares and ground squirrels. Hence, it has also to be considered in the differential diagnosis of plague. The disease is prevalent only in certain restricted areas in America, Japan, Russia, Norway and the Middle East. From the infected animals the infection may spread accidentally to man either directly through handling the infected animals or through the bites of ticks, flies and other blood-sucking arthropodes. The organism possesses a very high degree of infectivity and can penetrate

through the intact skin or mucous membrane; consequently, risks of laboratory infection are also very great. Due to its high invasiveness, septicaemia is very frequent, especially in the early stages of the illness. The organism does not produce any exotoxin.

Antibodies, especially agglutinins, are developed in the sick person and are present in the blood in high concentration. Hence the agglutination reaction may be employed for diagnosis. It should be remembered that cross agglutination reactions between *Past. tularensis* and *Brucella* organisms occur. Also, exudate from ulcers or gland puncture juice may be inoculated into guinea-pigs or mice which will develop the condition.

Pasteurella Pseudotuberculosis. This organism is responsible for a tuberculosis-like disease in rodents, especially the guinea-pig and rat. It resembles very closely the pestis bacillus, but unlike the latter, it is motile and flagellated at 18°–26° C. and tends to turn litmus milk alkaline. Another differentiating feature is its low pathogenicity for white rats. It shows marked cross agglutination with *Past. pestis.* A few cases of human infection with it have been reported. In any diagnostic inoculation of guinea-pigs, the possibility of a natural infection of the animal with this organism should be kept in view.

CHAPTER XXV

BRUCELLA

The organisms belonging to the genus *Brucella* are minute, non-sporing, usually non-motile, Gram-negative rods which may even appear coccoid, possessing no fermentative activities on carbohydrates. They are aerobic or microaerophilic. They are strict parasites, occurring in animals and man.

The following species of human and veterinary importance are contained in this genus: *Brucella melitensis*, *Brucella abortus*, *Brucella suis* and *Brucella bronchiseptica*. They are naturally parasitic on domestic animals, like sheep, goats, cattle and pigs: the first three species cause septicaemia and spontaneous abortion in these animals and undulent fever in man. Though classified as separate species, they display close similarity in their morphology, physiology and pathogenic properties. It is not unlikely that they had a common ancestor but have now come to show certain differences because of their long sojourn in, and adaptation to, different animal species. *Br. bronchiseptica* differs from the rest in being motile with peritrichate flagella. Originally described as the cause of canine distemper, it was subsequently shown to be only a secondary invader in this disease which is now accepted as viral in origin. The inclusion of *Br. bronchiseptica* in this genus is only tentative and may require revision in the light of future studies. The organism of tularaemia, *Past. tularensis*, is included in this genus by some workers owing to its close morphological and serological relationship with the *Brucella* organisms (p. 499).

Brucella Melitensis. *Br. melitensis* was first isolated by Bruce in 1887 from the spleen of patients suffering from an undulent type of fever (Malta fever) at Malta. It was subsequently shown to be a natural parasite of goats and sheep, in which it often causes a septicaemic type of disease with spontaneous abortion.

Ecology. It is a strict parasite, naturally occurring in sheep and goats. It is present in infected soil and pasture. The organism is also found in the lesions of Malta fever.

Morphology. *Br. melitensis* is a tiny delicate bacillus 0·6-1·2 μ long by 0·6 μ broad, with straight axis and rounded ends. Coccoid forms and longer forms up to 2 microns may be observed; coccoid forms are much more frequent in this than in the other species. Usually arranged as single discrete elements, short chains also may rarely be seen. It is non-motile and does not develop capsule or spores. It stains easily; it is Gram-negative; bipolar staining is common.

Growth Requirements. *Br. melitensis* is rather exacting in its nutritional needs and liver infusion seems to contain the necessary growth-promoting factors. From studies of its growth conditions in amino acid synthetic media, the organism is seen to require nicotinamide, thiamine and pantothenic acid. *Br. melitensis* is an aerobe; growth does not occur under strict anaerobic conditions. Though not necessary for growth, 10 per cent. CO_2 improves it. Although active multiplication occurs between 20° and 40° C., it does so best at 37° C. It grows well between pH 6·6 and 7·4.

Cultural Characters. Although growth occurs on all media, it is rather slow and never profuse. As mentioned above, better growth can be obtained on liver infusion agar. The addition of glucose also improves growth. The organism grows on MacConkey's agar. On agar small, round, convex, greyish colonies, about 0·5 mm. in diameter, are developed in 24–48 hours. Later a brownish coloration may develop. This is more marked on potato. The colonies are easily emulsifiable. Growth in broth is poor, causing but slight turbidity and late light deposit; no pellicle is formed. In old broth cultures the deposit is very viscous and difficult to break; the reaction is highly alkaline, pH 8·0 or higher.

Biochemical Reactions. It evinces no fermentative properties; no indole is formed, nor gelatin liquefied. Litmus milk is slowly turned slightly alkaline. Nitrates and often nitrites are reduced. Hydrogen sulphide is not formed. A brown pigment is elaborated on potato and often on old agar culture. No haemolysin is produced.

Resistance. *Br. melitensis* has only the ordinary resistance to injurious agencies. It perishes in ten minutes when exposed to

moist heat at 60° C. It is readily destroyed by pasteurisation. It has been found to remain alive in dust up to 44 days and in soil and water up to ten weeks or longer. It resists desiccation for two to three months. Cultures remain viable for several months. *Br. melitensis* resists the action of certain dyes, like basic fuchsine and thionin, in high dilutions, a property made use of in the differentiation of species. (Refer Table XVII).

Antigenic Structure. The *Brucella* organisms in their smooth phase possess two chief O antigenic components, designated *M* and *A*. Their exact chemistry is not known. The relative proportion of each of these varies in different species; the *M* fraction predominates in *Br. melitensis* and the *A* fraction in *Br. abortus*, whereas in the porcine type, *Br. suis*, they are more balanced with a slight preponderance of the *A* component. Rough variants do not show the presence of these antigens and are not agglutinable by antisera prepared against smooth strains.

Variation. Under artificial conditions the *Brucella* organisms undergo rather slow dissociation of the S–R type. The transformation is associated by a gradual loss of immunological specificity and virulence and a progressive acquisition of the property of non-specific agglutination. The non-recognition of this fact has led in the past to the erroneous creation of separate serological types, like *Paramelitensis* and *Paraabortus*, which were really

TABLE XVII

Classification of *Brucella* Group

Species	CO_2 requirement	H_2S production	Growth in presence of		
			Basic fuchsine 1: 25,000	Thionin 1: 30,000	Methyl violet 1: 100,000
Br. melitensis ..	−	−	+	+	+
Br. abortus ..	+	+	+	−	+
Br. suis ..	−	±	−	+	−

± = variable; the American type produces H_2S but not most of the strains found in Denmark.

strains in the transition and rough phases, giving partial or no agglutination with sera prepared against smooth strains. These points are of great practical significance and should be kept in mind when doing identification tests with sera, in order to avoid false positives due to spontaneous agglutination. It is imperative that only known smooth cultures are employed in serological tests.

Toxin Production. No diffusible toxin is synthesised by this organism. The endotoxin is presumably responsible for the toxic effects.

Pathogenicity. Animals are the primary victims of *Brucella* infection and from them man is secondarily infected. These organisms are highly invasive to their respective hosts. *Br. melitensis* appears to be the most and *Br. abortus* the least infective to man. *Br. melitensis* causes a natural septicaemic infection in goats, sheep and rarely cattle and undulent fever (Malta fever) in man. Its infectivity to man is very high.

Experimentally sheep and goats can be readily infected and the disease so induced exhibits the same clinical course as the natural disease. Spontaneous abortion is the most obvious clinical manifestation of the infection, experimental or natural, although it is not necessarily an invariable event. Experimental infection of the common laboratory animals may not be so successful as in domestic animals, but guinea-pigs, rabbits, rats, mice and monkeys may be infected; of these, the guinea-pig is the most susceptible. The reports regarding the relative virulence of the different *Brucella* strains to the laboratory animals are conflicting. To the guinea-pig, probably, *Br. abortus* is the least and *Br. suis* the most virulent. *Br. abortus* is less virulent to monkeys than *Br. melitensis*, whereas the swine type resembles the latter in this respect. In monkeys *Br. melitensis* causes an almost identical condition like Malta fever.

Brucella Abortus. *Br. abortus* (Bang, 1897), the organism of contagious abortion in cattle, is morphologically indistinguishable from *Br. melitensis*, except that it is slightly longer and more bacillary.

A high carbon dioxide tension, 5-10 per cent., is obligatory for the growth of *Br. abortus*; but by serial subcultures it can be trained to grow in the ordinary atmosphere. It is inert on sugars but produces a brownish pigment on potato. The abortus

bacillus and some of the porcine strains, unlike the melitensis bacillus, produce hydrogen sulphide. This can be determined by the use of lead acetate paper. *Br. abortus* is inhibited by thionin but not by fuchsine, whereas *Br. suis* is inhibited by fuchsine but not by thionin. (Refer Table XVII).

Br. abortus causes contagious abortion in cattle and mares and infects humans in whom no abortion but an undulent type of fever, called abortus fever, is caused.

Br. abortus gives rise to tubercle-like lesions in the guinea-pig. The organism is also present in the milk of infected cows. These facts are of importance and, unless kept in view, mistakes are likely to be committed when testing milk for the presence of the tubercle bacillus by guinea-pig inoculation.

Brucella suis. Two types of porcine strains, the American and the Danish, have been described. In morphology they resemble the abortus type. Thionin does not suppress the growth of both types, but fuchsine does. Growth is not improved by carbon dioxide. While the American type produces hydrogen sulphide, the Danish type does not. *Br. suis* is a strict parasite, occurring in pigs and man. The porcine type of undulent fever is more serious than the cattle type. *Br. suis* may rarely infect cows.

Brucellosis is primarily a disease of animals, *Br. melitensis* being responsible for it in goats and sheep, *Br. abortus* in cattle and mares and *Br. suis* in pigs. *Br. melitensis* infection has also been reported in cows and *Br. abortus* in sheep and goats. In these natural hosts, the disease is septicaemic in nature and may cause abortion in the female. The disease may also be chronic or the infection may remain inapparent. The excretion of organisms from the infected animal occurs in milk and urine. It may be sudden, transitory or lasting for weeks. The organism is found in large numbers in the uterine discharges of aborted animals. Through these manure and pasturage may be readily infected and in them the organism can remain viable for weeks. This is probably the chief method of spread among animals.

Undulent fever is a world wide disease and is found only in association with sheep, goats, cattle and pigs. But its prevalence is not so extensive as one would suppose from the large numbers of these animals existing everywhere. It is present in this country, but to a limited extent. Human infection from pigs is very rare. There is a seasonal fluctuation in the incidence of undulent fever,

the peak period corresponding to the months of lambing or calving and abortion. The spread of infection to man may occur in several ways; the consumption of infected milk and milk products is the most common. As in animals, man is also readily infected *via* the alimentary tract. Contact with the infected animals, dead or alive, and infected manure may serve to transmit infection. Hence, butchers and other employees of slaughter houses, veterinary doctors and attendants and farmers are particularly liable to infection by this means. This is the more important mode of spread in the porcine type. Rarely infection may be contracted through handling cultures in the laboratory; many cases of such infection are on record. The infection can probably pass through the unbroken skin. The disease is probably more frequent in the rural than in urban areas. Infants and children are less affected than adults; females seldom get the infection, probably because opportunities for contracting it are less in them.

The incubation period is not definite; according to some it may be from 8 to 14 days, while others report that it may vary from a few days to several months. Malta fever is more serious than abortus fever; it is also more infective. Latent infection with *Br. abortus* seems to be common, as judged by the presence of agglutinins in the blood of persons with no history of abortus fever, particularly in those coming in contact with cattle. The porcine type is less frequently transmitted to man but almost equally virulent to him as *Br. melitensis.* Undulent fever due to abortus infection is the least serious of the three. The *Brucella* organisms are very invasive and undulent fever, as a rule, is a generalised infection; the organism can be isolated from the blood, especially in the early stages of the illness and during the period of rising temperature in a pyrexial bout. It is also excreted in the urine. Malta fever has a particularly prolonged course. The mortality is low even in the more serious melitensis type; may be about 2–3 per cent.

Immunity. Antibodies, like agglutinins and immune opsonins, are developed during the disease. An allergic sensitisation also develops; it is more pronounced in this infection than in many other bacterial infections. Recovery from an attack is followed by immunity of fairly solid and lasting nature, but subsequent attacks may occur.

Diagnosis. The laboratory diagnosis of undulent fever is rather difficult. It is based on the isolation and identification of the organism from the blood or urine and on the demonstration of specific antibodies in the patient's serum. The latter is by no means conclusive.

Blood culture must be done during the course of a pyrexial wave, preferably during its height. As the organism may be relatively scanty, not less than 10 c.c. of blood should be withdrawn and 5 c.c. added to each of two flasks containing 100 c.c. of glucose broth or liver infusion broth; one is incubated under ordinary aerobic condition and the other in 10 per cent. carbon dioxide tension. Growth is slow and may take several days; hence subcultures at 2–5 days interval should be done and no negative report should be sent before three weeks. For subculture liver infusion agar or tryptose agar may be used. The culture of material from spleen puncture may likewise be attempted The organism is excreted in the urine, but it is intermittent. Hence, urine should be repeatedly cultured for three days during the ascending phase of fever, using morning specimens. Gentian violet, added to the medium in a concentration of 1 : 100,000, will help in the isolation of the abortus bacillus by suppressing the growth of other bacteria. The identification of the isolated organism is based on its carbon dioxide requirement, hydrogen sulphide production, resistance to dyes and serology. Where possible monospecific serum should be used in the serological identification.

Serological Methods. Agglutinins are usually found in blood in appreciable titres by the fifth to eighth day after the onset of the illness. Their concentration increases rapidly and titres above 1 : 640 are quite common; often 1 : 5120 or higher is obtained. Hence high dilutions of the patient's serum should be put up. It may be remembered that the bacterial antigen should be prepared from a smooth culture. The occurrence of prozone phenomenon is very common in this and should be kept in view when reading the result. Positive agglutination results are often obtained earlier than positive cultures owing to the slowness of growth.

Normal people may show in their blood agglutinins against the *Brucella* organisms up to a titre of 1 : 25. Normal contacts, like breeders of livestock, veterinarians, butchers and dairy

workers, may contain agglutinins up to a titre of 1 : 100. These may cause confusion. Further, patients not seldom develop group agglutinins almost to the same titre as the specific agglutinins. When it occurs, absorption tests have to be done to establish the diagnosis. A small proportion of patients entirely fail to develop agglutinins throughout the course of the disease, and so a negative reaction does not always exclude *Brucella* infection. Again, in certain diseases, like typhoid fever, tuberculosis and tularaemia, agglutinins have been found to develop against the *Brucella* organisms. Furthermore, anamnestic rise in titre may also occur. Thus, all the difficulties encountered in the interpretation of the Widal results are likewise present in the interpretation of the results of agglutination test in brucellosis. From what has been said above it will be obvious that a titre of 1 : 100 or less has little diagnostic value. A progressive increase in the titre is the surest test and should be the guide. However, a fairly high titre for one type, such as 1 : 320, may be taken as diagnostic, when the titres for the other types are relatively low or absent.

The complement-fixation and precipitation tests may be positive, but they are impracticable as routine measures. An intradermal allergic test, *brucellin* test, has been developed: the antigen employed is the filtrate from a twenty days' broth culture after sterilisation by heat; 0·1 c.c. of it is inoculated intradermally; in positive cases a local reaction, consisting of erythema and induration, occurs in 24–48 hours.

Diagnosis in Animals. The organisms are found in large numbers in the uterine discharges and aborted factuses, less in milk, blood and urine. The isolation of the offending microbe from these materials and its identification should be followed on the same lines as described above. If the animal is dead, culture of spleen pulp may also be done. The addition of gentian violet, 1 : 100,000, to the medium (with a pH of 6·6) will greatly facilitate the isolation of *Br. abortus* by a selective inhibition of the concomitant organisms: the culture should be incubated in an atmosphere of 5–10 per cent. carbon dioxide tension. The intraperitoneal inoculation of guinea-pigs with suspected milk may be resorted to for the isolation of the organism. The animal is killed after a month and cultures made from the blood, spleen or other internal organs. Agglutination test with the animal's

serum and milk (Zammit's test) and the abortin test are also helpful.

Treatment. Vaccine treatment may be tried but is of doubtful value. Treatment with antiserum has not yielded encouraging results. The dose is 50 c.c. per day, repeated for five or six days. Sulphonamide does not hold out much hope in the treatment of undulent fever.

Prophylaxis. The destruction of infected animals or their segregation from the herd may help to reduce the incidence of the disease. General methods are avoiding contact with infected animals and adequate sterilisation of milk by pasteurisation or boiling. The latter is probably the surest method. Prophylactic vaccination has been tested with unsatisfactory results. A live attenuated vaccine has been tried in the immunisation of cattle, but it is too risky for man.

Brucella Bronchiseptica. This organism is hardly distinguishable morphologically from *Br. abortus*, except that it is motile by means of peritrichate flagella. It is anaerobic and does not produce hydrogen sulphide. It forms a haemolysin. *Br. bronchiseptica* is serologically related to the other *Brucellas* but can be separated from them by the agglutinin absorption technique. Morphologically and serologically it is also closely related to *H. pertussis* and so some authorities place it in the genus *Haemophilus*. It was originally isolated from canine distemper and considered for a long time as its cause. But now it is regarded only as a secondary invader in this disease, the primary cause being a virus. It causes bronchopneumonia in guinea-pigs and rodents.

CHAPTER XXVI

HAEMOPHILUS

The *Haemophilus* organisms are minute, rod-shaped cells, often markedly pleomorphic, non-motile, non-spore-forming and Gram-negative. They are strict parasites, requiring, in general, serum or ascitic fluid for growth. Some species also require for growth certain accessory food factors present in haemoglobin and some vegetable tissue (see below).

This genus contains several species of medical interest: *Haemophilus influenzae* including the haemolytic strains which are sometimes classified as a separate species—*H. haemolyticus*; *Haemophilus parainfluenzae*, found in the upper respiratory tract of man and cat, requires the V but not the X factor for growth; and *Haemophilus pertussis*, the causative organism of whooping cough. Two other closely related species, *Haemophilus suis* (*H. influenzae suis*) and *Haemophilus canis* (*H. haemoglobinophilus*), are found in domestic animals. Ducrey's bacillus of soft sore, *Haemophilus ducreyi*, and the Morax-Axenfeld bacillus, *Haemophilus duplex*, are also included in this genus. In view of the fact that some of these species will grow in the absence of haemoglobin, no real justification can be given for their inclusion in this genus. Many authorities do not consider Koch-Weeks bacillus as distinct from *H. influenzae*; Bergey uses these terms synonymously. Allusion has been made in a previous chapter that some authorities include *Br. bronchiseptica* in this genus because of its close similarity to *H. pertussis*.

Haemophilus Influenzae. *H. influenzae* was first isolated by Pfeiffer in 1892 from the sputum of influenza patients and it was for a long time regarded as the cause of influenza. It is commonly known as Pfeiffer's bacillus. As its causal relation to influenza has been disproved, the name *H. influenzae* is misleading and requires revision.

Habitat. It is a strict parasite found in the normal upper respiratory tract of man and in lesions caused by the organism. It

should not be confused with an identical parasite found in the respiratory tract of pigs, *H. suis*, or with another present in the preputial secretions of dogs, *H. canis*.

Morphology and Staining. The influenza bacillus is one of the smallest pathogenic bacteria. The cells are very tiny, slender rods, $1 \cdot 5 \mu$ long by $0 \cdot 3 \mu$ thick, with rounded ends and occurring in singles, pairs and occasionally in short chains. Pleomorphism is a marked feature, showing at times long bacillary and thread forms. This irregularity is a striking feature of this species. The strains isolated from pathological lesions show a preponderance of the coccobacillary forms. And some people regard these forms as the typical and the longer strains as the atypical forms. The organism is non-motile and does not form spores. Some strains are capsulated. *H. influenzae* does not take stain readily. Prolonged staining with dilute carbol fuchsine gives the best result. Both in the Gram's as well as in the acid-fast method the primary stain is lost.

Growth Requirements. *H. influenzae* is aerobic and facultatively anaerobic. Some strains are incapable of growing under anaerobic conditions. Active growth and multiplication occur between 26° and 43° C., the optimum temperature is 37° C.

Pfeiffer's bacillus is a fastidious organism and requires two accessory factors, X and V, for its growth and metabolism. These essential growth-promoting substances are present in whole blood and certain plant tissues. The V factor, so called because of its similarity to vitamin, is found plentifully in yeast and potato. It is destroyed by heat at 120° C. in 30 minutes, whereas the X factor withstands this temperature. The V substance is also formed by many organisms, for example *Staph. aureus*. The X factor is contained in haematin or is probably identical with it. These factors probably play a part in the respiratory processes of the microbe. The role of the X substance is probably in the synthesis of peroxidase and catalase. The atypical strains require only the V factor for growth. Because of its fastidious nature, Pfeiffer's bacillus will not grow on ordinary media. The addition of blood to these will promote growth, as indicated above, because it contains both the X and V factors. Heated blood is preferable, since by this haemoglobin is converted into haematin, thereby enhancing its value for growth. Heating blood agar for one to three minutes at 100° C. brings about this result. In

Fildes' medium blood, previously digested with pepsin, is added to agar or broth.

Cultural Characters. On heated blood agar medium (chocolate agar) the organism develops into small, pin point, discrete, transparent colonies. In the neighbourhood of staphylococcal colonies, Pfeiffer's bacillus grows luxuriantly, developing into larger more opaque colonies, *satellitism*, owing to the fact that the staphylococcus elaborates the V factor during growth and liberates it into the surrounding medium. Growth in fluid media results in slight uniform turbidity. The atypical forms may give a flocculent deposit with little or no turbidity.

Biochemical Activity. The activity of *H. influenzae* in this respect is limited; it has not been fully studied. Acid without gas is produced in glucose; maltose, saccharose and laevulose are irregularly attacked, probably more frequently by rough and atypical strains. Mannite and lactose are never fermented. The typical strains (about 40 per cent.) produce indole. The haemolytic strains are practically always non-indole-producers.

Resistance. The usual antiseptic agents kill the influenza bacillus readily. Heat at 55° C. destroys it in thirty minutes. Cultures die out soon and for preservation subcultures must be made every four or five days. *H. influenzae* withstands the action of penicillin and in fact, it may be recalled, this was one of the earliest observations made regarding the resistance of certain organisms to penicillin.

Antigenic Structure. The members of this species do not constitute a homogeneous group. They fall into two subgroups—the smooth and the rough. Six serological strains, named from *a* to *f*, are recognised among the smooth type; most of them are capsulated and specificity among them seems to depend upon a polysaccharide surface antigen, probably of the capsule. The strains isolated from cases of influenzal meningitis, which are generally very virulent, are usually of type *b*. As indicated by cross agglutination, some of these types are immunologically related to certain types of pneumococci. The rough type is far more commonly present in the normal throat than the smooth type; the former is also antigenically heterogeneous. A protein substance, common to most strains of *H. influenzae*, has been recently described—the M fraction.

Toxin Production. No diffusible toxin is elaborated by the influenza bacillus. The cell substance is presumably the toxic factor.

Pathogenicity. Experimental inoculation into monkeys has been reported to be successful, producing an acute respiratory condition resembling influenza, when the organism is introduced through the nose. Similar experiments on man have, however, proved largely unsuccessful. Massive doses, administered into the peritoneum of the usual laboratory animals, will kill them in 24–48 hours, probably by toxaemia.

H. influenzae produces natural infection only in man. The primary cause of epidemic influenza is a virus, and *H. influenzae* probably plays only a secondary role in its pathogenesis. It is considered to be responsible for the secondary inflammatory lesions occurring in this disease. The organism is found in large numbers in the sputum and the respiratory discharge of a large majority of cases of influenza. In association with the streptococcus, pneumococcus and other pathogens, Pfeiffer's bacillus occurs in non-influenzal inflammatory and catarrhal lesions of the respiratory tract and lungs, such as bronchopneumonia, abscess, empyema, bronchiectasis and others. Not infrequently, it is present in diseases like whooping cough, measles and tuberculosis. How far its presence in these various lesions is but a mere reflection of its normal existence as a parasite in the respiratory tract or to what extent is it contingent on the virulence of the organism, is not clear. Probably its virulence can have play only when there is a previous lowering of local resistance. This germ is also associated with sinusitis, otitis media, meningitis and ulcerative endocarditis. Meningitis due to this organism is very fatal; the cellular response is often meagre and large number of bacilli are present in the cerebrospinal fluid, often in clumps. The occurrence of influenzal meningitis indicates that the organism can independently cause disease in man.

H. influenzae also causes epidemic catarrhal conjunctivitis. No separate description of Koch-Weeks bacillus is necessary as there is no point of difference between it and Pfeiffer's bacillus. The eye strains do not grow in the absence of the X and V factors. The organism was first observed in the eye lesions by Koch in Egypt and Weeks in New York. It is now accepted as the cause of one type of epidemic catarrhal conjunctivitis. It is found

in large numbers in the lesions and discharges, both extracellularly and intracellularly. Transmission is through direct contact with infected hand, towels, handkerchiefs and the like.

Immunity. Not much is known about immunity against *H. influenzae*. Antibodies, like agglutinins and complement-fixing antibodies, are produced and found in the blood in infections with this organism.

H. parainfluenzae is another related species closely resembling the influenza bacillus; it is also present normally in the upper respiratory tract of man. It has been isolated from some of the above mentioned lesions; many of the strains isolated from endocarditis were of this type.

H. suis or the swine influenza bacillus, in conjunction with a virus, appears to be responsible for swine influenza. It resembles Pfeiffer's bacillus and like this requires both X and V factors for its growth. Though *H. canis* also resembles *H. influenzae* in several respects, it can grow in the absence of the V factor but not of the X factor.

Diagnosis. Stained smears of sputum or discharge show the fine Gram-negative bacilli in large numbers, often within the polymorphonuclear cells. Finding these in the exudate from eyes in conjunctivitis or in the cerebrospinal fluid in meningitis is practically enough for diagnosis. Dilute carbol fuchsine is the stain of choice. Culture of the organism on chocolate agar or Fildes' medium, followed by its isolation and identification, may be proceeded with, when considered necessary. Penicillin, by inhibiting the growth of other organisms, is useful for rapid isolation and may be employed.

Treatment. Pfeiffer's bacillus is also included by some workers in vaccines prepared in the treatment of chronic respiratory infections. Its usefulness has not been established.

Haemophilus Pertussis (Bordet-Gengou Bacillus). *H. pertussis* (L., whooping cough) was isolated by Bordet and Gengou in 1896 from the sputum of cases of whooping cough.

Morphology. The pertussis bacillus is slightly thicker and more oval than the influenza bacillus, $1 \cdot 5 \times 0 \cdot 5 \mu$. It is also much less pleomorphic than *H. influenzae* and does not show the latter's tendency to form thread-like and filamentous forms. Freshly isolated strains may show capsule. It is non-motile and non-sporing. Staining is not easy and the same method has to

be used as in the staining of Pfeiffer's bacillus (q.v). It is Gram-negative and frequently shows a tendency to polar staining.

Growth Requirements. It is a strict aerobe. The optimum temperature is 37° C. It is less haemophilic than *H. influenzae*. Neither the X nor V factor is necessary for its growth.

Cultural Characteristics. *H. pertussis* does not grow on ordinary media. The special medium devised by Bordet and Gengou is necessary for at least primary culture. Even on this growth is slow; the colonies appear as small, round, greyish white and dome-shaped, resembling split pearls or mercury drops. The growth is sticky and tenacious due to the presence of a mucoid substance. On blood agar the colonies are surrounded by a narrow zone of haemolysis. By repeated subculturing the organism can be trained to grow on ordinary media. After adaptation, growth in broth gives rise to turbidity and later heavy, ropy, sediment and floating strands.

Biochemical Activity. The pertussis bacillus is biochemically inert. It has no action on carbohydrates. It does not form indole nor liquefy gelatin. It is weakly haemolytic.

Resistance. Its resistance to adverse agencies is not high. Heat at 55° C. kills the pertussis bacillus in 30 minutes. Unlike *H. influenzae*, it is fairly resistant to cold. Penicillin does not inhibit its growth and this property may be utilised to prepare selective media for its isolation.

Antigenic Structure. *H. pertussis* in the normal smooth phase is immunologically homogeneous. Four phases, I, II, III and IV, have been described. These represent probably successive stages in the way to rough dissociation. Serologically there is not much difference between *H. pertussis* and *H. parapertussis* recently described from the late stages of whooping cough. The pertussis bacillus displays serological affinity with *Br. bronchiseptica*.

Variation. Strains freshly isolated during the catarrhal stage are in the normal smooth phase. On subculture they undergo dissociation. This is accompanied by loss of virulence and changes in antigenicity. Four phases have been described as occurring in the course of such dissociative changes (see above). The final rough variants are non-haemolytic, non-capsulated and non-virulent. Strains isolated during the later stages of the disease (the so-called *H. parapertussis*) may not be entirely in the normal smooth phase.

Toxin Production. *H. pertussis* does not produce a soluble toxin. A powerful endotoxin is formed. It can be separated as a watery extract from culture. Evidence has been brought forward to show that it is made up of two components, a heat stable one and a heat labile one which is inactivated at 56° C. in thirty minutes. The endotoxin is commonly believed to be what is concerned in pathogenesis. It proves fatal to guinea-pigs when administered intravenously. In man it causes positive skin reactions probably as the result of acquired allergy. The mucoid exudate responsible for the whooping is ascribed to a separate toxic factor developed by the organism only in its less virulent phases.

Pathogenesis. Bordet and Gengou's view that *H. pertussis* is the cause of whooping cough is now generally accepted, although it has been severely criticised by many workers. The pertussis bacillus is constantly present in the disease, in large numbers in the early catarrhal stage and less frequently in the paroxysmal stage, disappearing progressively during the decline; it is seldom found after the fourth week of disease. Except probably in contacts, it is not found in the healthy subject. The results of attempts to reproduce the disease in experimental animals and human volunteers are suggestive but not conclusive. The intratracheal inoculation of mice or the intranasal inoculation of rats has been found to produce an interstitial pneumonia with leucocytic infiltration around the vessels and bronchioles and copious secretion of the bronchial mucosa. A condition somewhat resembling whooping cough has been produced in chimpanzees and human volunteers by the intranasal instillation of cultures. Large doses of culture injected into experimental animals cause intense toxaemia and death.

Pertussis is a highly communicable disease. As noted above, secretions from the nose and the respiratory tract contain the organism in large numbers in the early stages of the disease. Transmission is by direct contact and by droplet infection. As the pertussis bacillus does not survive long outside the body, indirect transmission by the agency of fomites may occur only very rarely. The disease is most infectious during the catarrhal stage before the whoop begins. After this, the danger of transmission progressively falls to a negligible degree by the fourth week. Undiagnosed and atypical cases may also play a part in the dissemination of the infection. The existence of healthy

carriers of the pertussis bacillus has not been proved. *H. pertussis* is not invasive and is never found in blood culture. The infection is confined to the upper and lower respiratory tract but causes systemic effects, including fever and the well known lymphocytosis of pertussis, from toxaemia.

Whooping cough is a world wide disease. It is common in the tropics but not so fatal as in the cold countries where it is one of the major killing diseases of children. Infants under three months of age usually escape the disease. There is no proof that this is due to any placental transfer of maternal antibodies, as in diphtheria, measles and scarlet fever. The most susceptible period, and the one with the highest fatality, is from six months to three years. More than 80 per cent. of cases occur in infants and children up to the age of five years. It is said to be somewhat more frequent in females than in males. The incubation period varies from a few days to a fortnight. The average mortality rate is 15 per cent.

Immunity. Agglutinins and complement-fixing antibodies are found in the blood but late in the disease, so that they are not of much diagnostic help. Immunity against pertussis is acquired with increasing age, probably as the result of subclinical infection. The immunity conferred by one attack seems to be solid and lasting. But second attacks have been reported.

Diagnosis. During the first two weeks, smears from the sputum or nasal secretions usually show the organism. The cough plate method of cultivation affords the greatest success; sputum culture is much less satisfactory. In the former there is direct implantation of the secretions from the respiratory tract into the medium. A plate of the Bordet-Gengou medium is held for fifteen seconds at a distance of four to five inches in front of the patient's mouth while coughing. Excessive coughing and exposure may both cause overcrowding of colonies. The addition of penicillin to the medium may help by suppressing most of the associated organisms. After incubation, subcultures are made from the pearl-like grey colonies. No plate should be discarded as negative before five days. The isolated organism is then identified microscopically, culturally and by the agglutination test with the specific serum.

With the patient's serum agglutination and complement-fixation reactions may be positive by the third or fourth week.

The antigen employed should be a killed suspension of the pertussis bacillus in the smooth phase. These tests are useful after the third week when culture is difficult.

Prophylaxis. Early diagnosis and immediate isolation are very important in prophylaxis. The ideal is to isolate in quarantine all susceptible contacts for fourteen days. Until two successive cough plates are negative or up to the end of the third or fourth week after the onset of the characteristic whoop, the patient is assumed to be communicable.

Active immunisation has been found to be a valuable preventive measure. The disease is relatively infrequent or milder in the immunised children. Immunisation must be done between six and twelve months of age. Vaccine must be prepared from freshly isolated virulent strains; early failures were very likely due to the employment of dissociating strains in the preparation of vaccine. Large doses are necessary. Usually vaccine is sterilised with 0·5 per cent. phenol and is standardised to contain 10,000 million bacilli per cubic centimetre. Four subcutaneous injections, 1 c.c., 1·5 c.c. 1·5 c.c. and 3 c.c., are considered adequate. It has been advocated recently to immunise simultaneously against pertussis and diphtheria by combining the pertussis vaccine with diphtheria toxoid.

Passive immunisation by the injection of 10–20 c.c. of pooled convalescent or immune adult serum may have some prophylactic value. But neither vaccine nor antiserum has any therapeutic utility; their use has given only disappointing results.

Haemophilus Duplex (Morax-Axenfeld Bacillus). Morax-Axenfeld bacilli are non-motile, non-sporing, non-capsulated, Gram-negative rods. In films from the eye, they are found in large numbers, 2–3 microns by 1 micron in size, rather plump with rounded ends and occurring in pairs; they may be extracellular or intracellular. Culture forms are smaller and may be discrete, paired or in short chains.

It is aerobic and facultatively anaerobic; the optimum temperature is 37° C. Blood or serum is required for its growth. On blood agar plate the organism grows in small, circular, transparent colonies. Delayed, but definite, liquefaction occurs around colonies on Loeffler's medium. Growth in serum broth causes general turbidity.

Indole production is negative. Various carbohydrates are attacked. Coagulated serum is liquefied.

H. duplex is associated with subacute or angular conjunctivitis. It is also invariably present in angular stomatitis. How far it is the real causative agent in these conditions, has not been determined.

Petit's bacillus is another diplobacillus found in chronic conjunctivitis. It is morphologically identical with *H. duplex*, but smaller: it grows well on ordinary media.

Haemophilus Ducreyi. Ducrey's bacillus, as found in stained smears from gland discharge, is a small, ovoid, rod-shaped organism, arranged in pairs side by side, groups or in chains, about $1 \cdot 5 \mu$ by $0 \cdot 4 \mu$ and with rounded ends. Bipolar staining may be seen. The organism may be intracellular. It is non-motile, non-sporing, non-capsulated and Gram-negative.

Cultivation is difficult, and enrichment with blood or serum is necessary for growth. Neither the X nor V factor is essential for growth; hence its inclusion in the genus *Haemophilus* is open to question. Uncontaminated material obtained by puncturing a bubo should be directly inseminated into media.

It is associated with soft sore or chancroid, a veneral disease. Transmission is usually by sexual contact. An identical lesion has been experimentally produced in man and higher monkeys by the inoculation of pure cultures. Ducrey's bacillus is not pathogenic for lower animals. Little or no immunity develops as the result of an attack and subsequent attacks are common. An intradermal skin test, employing killed culture, is used for diagnosis; it is based on allergic sensitisation; 0·1 c.c. intracutaneously administered is the dose. An antigen prepared from aspirated pus, as in the case of Frei's antigen, may also be employed. The hypersensitivity lasts for several years.

CHAPTER XXVII

CORYNEBACTERIUM

This genus includes several species of medical and veterinary importance. The individual cells show a tendency to the formation of club-shaped swellings at the ends, hence the name *Corynebacterium*. They are Gram-positive, slender and often slightly curved rods which are non-motile, non-sporing and aerobic or microaerophilic. Characteristic snapping motion is shown when cells divide. None of the members seems to lead a free-living existence. Some species produce a powerful extracellular toxin and many are pathogenic.

Diphtheria as a clinical entity distinct from other throat affections was separated by Bretonneau as early as 1826, but several decades had to elapse before its aetiology was discovered. Klebs (1883) was the first to observe and describe what was later confirmed as the diphtheria bacillus; he found it in the pseudomembranes from the throats of diphtheria cases and asserted that it was the cause of diphtheria. In the following year the same organism was isolated in pure culture from a number of cases of diphtheria and fully described by Loeffler. By inoculating the isolated organism upon the abraded mucous surfaces of susceptible animals, he was also able to reproduce lesions closely resembling the false membrane of the human disease. Subsequently, several others confirmed the constant presence of it in the characteristic false membrane of diphtheria. Its aetiological relationship to the disease was finally established by the work of Roux and Yersin (1888) who showed that this organism elaborated a soluble toxin which was capable of reproducing certain characteristic symptoms and lesions of diphtheria experimentally. Although *Corynebacterium diphtheriae* is now accepted as the type species, the xerosis bacillus was probably the first member of the group to be discovered.

The term "diptheroid" (diphtheria-like) is frequently applied to the other members of the genus, as they bear a close morpho-

logical resemblance to the diphtheria bacillus. Numerous species have been described, many of which are devoid of any pathological significance. The species commonly associated with man are *Corynebacterium hofmannii* (*Corynebacterium pseudodiphtheriticum—Bergey*), *Corynebacterium xerose* and *Corynebacterium acnes*. Their importance is mostly in connection with the differential diagnosis of *C. diphtheriae*. There are also a few important animal pathogens. *Corynebacterium ovis* or the Preisz-Nocard bacillus is a diphtheroid which causes caseous lymphadenitis and pseudotuberculosis in sheep and horses; *Corynebacterium pyogenes* is responsible for the production of certain suppurative lesions in pigs, cattle and other animals; *Corynebacterium enzymicum*, *Corynebacterium renale and Corynebacterium murisepticum* are other pathogens of animals.

Corynebacterium Diphtheriae. *C. diphtheriae* or the diphtheria bacillus is the causative organism of diphtheria. Though it was first observed by Klebs, we owe it to Loeffler for its isolation in pure culture and full description. Hence it is also known as Klebs-Loeffler bacillus.

It is a strict parasite found in the diphtheritic lesions in the tonsils, pharynx, nose and other sites and in the nasopharynx of carriers. It may be found in dust of rooms occupied by patients.

Morphology and Staining. They are slender, straight or slightly curved rods, occurring in singles or as small irregular clusters and varying greatly in size from 1·0–8·0 × 0·3–0·8 micron. The ends are rounded, rarely pointed, and frequently show a tendency to swelling and later club formation. Marked pleomorphism is a characteristic feature. True branching is occasionally seen in old cultures. In culture involution forms soon begin to appear, which are pear-shaped, club-shaped, globoid, fungoid or even streptococcal. Hence, an young culture, an eighteen- to twenty-four-hour growth, should be employed for the study of morphology. The characteristic staining reactions are also best brought out when the organism is grown on a medium containing serum, such as Loeffler's medium. The diphtheria bacillus does not develop flagella, spores or capsule.

When seen in film preparations, the arrangement of the bacilli is characteristic. They are grouped in small irregular clusters, in which each cell takes up an angular or parallel position in relation to its neighbour. This peculiar disposition resembles

Chinese letters or cuneiform writing. It appears to depend on the snapping mode of cell division. In this the parent cell begins to divide at one side and then breaks incompletely, the unbroken portion on the opposite side acting as a hinge-like process; consequently there is no separation of the daughter cells for a time.

Another distinctive feature is the presence of Babes-Ernst bodies or metachromatic granules (p. 5). Due to their presence the organism is unevenly stained. In young cultures of *C. diphtheriae* these granules can be readily demonstrated, but this is by no means the case with the allied diphtheroids. This serves as an important criterion in the differentiation of species. Anything up to six granules may be present in a single cell, but usually two or three. When only two are present they are most often seen one at each pole of the cell and are then termed "bipolar". When the number exceeds two, they lie scattered throughout the length of the cell, giving rise to a beaded appearance. It has been pointed out that the typical granular appearance is not found in all the strains of *C. diphtheriae* (*vide infra*). In order to show up the granules well, special staining methods are employed; the most satisfactory ones are Neisser's differential stain, Pugh's toluidine blue and Loeffler's methylene blue. By the Neisser's method, the granules are stained blue black standing out from a light brown cell body.

The diphtheria bacillus is easily stained by the coal-tar dyes. It is Gram-positive, but not so strongly as most other Gram-positive bacteria; the granules, however, are very resistant to decolorisation and remain deeply stained in contrast to the weakly stained protoplasm. Some of the diphtheroids, for instance Hofmann's bacillus, are strongly Gram-positive and this serves as a useful guide in the identification of species. The organism is not acid-fast.

Growth Requirements. *C. diphtheriae* is an aerobe and facultative anaerobe, but anaerobic growth is very sparse. The optimum temperature is 34°–36° C., but growth occurs well at 37° C. with a range of 15°–40° C. The optimum pH is 7·2. An acid reaction inhibits toxin production. The diphtheria bacillus is not very fastidious in its nutritional requirements and can be grown on ordinary media. But enrichment of these with serum promotes better growth; Loeffler's serum medium is an excellent one for its growth. The members of the *Corynebacterium* group

reduce tellurite and form greyish black colonies in media containing this chemical. On the basis of this a variety of differential media have been devised of late, all of which contain potassium tellurite. The better known of these are the tellurite blood agar medium of Anderson and his co-workers and Clauberg's medium.

Cultural Characters. In broth the growth is not uniform; some strains produce a granular deposit and a surface pellicle but no general turbidity; others produce a diffuse growth with uniform clouding and little or no deposit. After twenty-four hours' growth on Loeffler's medium, the colonies are about 1 mm. in diameter; they are circular with entire edge and convex, smooth or finely granular moist surface; the colour is greyish white or creamy; the consistence is butyrous and the colonies are easily emulsifiable. On further incubation the margin becomes crenated and the central portion more prominent and opaque than the periphery, while the colonies themselves become larger and show in some cases a distinct yellow color.

The tellurite blood agar medium is very useful not only in the isolation of the organism but also in the differentiation of types. When grown on this special medium, three types of colonies are encountered. These types are found to possess certain distinct biological characters and are associated with diphtheria of a greater or less clinical severity (p. 525). Hence the names *gravis*, *mitis* and *intermedius* have been given to them. It should be remembered that the organism, when grown on tellurite media, fails to show the characteristic morphology, for the study of which, therefore, the growth on Loeffler's serum should be employed.

The *gravis* type grows on tellurite medium in relatively large dark grey colonies with granular centre and striated or crenated periphery, often compared to the daisy head. Growth in broth gives rise to granular deposit and pellicle formation without general turbidity; there is an early reversal of pH in this medium. This type does not show the usual granular appearance but shows a lightly stained protoplasm with one or more deeper stained areas at the ends. Only about 50–60 per cent. of the gravis strains conform to this typical morphology; the rest resemble the *mitis* and the *intermedius* forms. It ferments starch, glycogen and dextrin; it is not usually haemolytic.

The *mitis* type develops into small, black, convex, smooth colonies with entire edge. In broth it causes diffuse growth and rarely pellicle formation; there is a late reversal of pH. It is this type that gives the characteristic granular staining; about 5–20 per cent. of strains, however, show barred appearance. It does not attack starch and glycogen and attacks dextrin irregularly; it is haemolytic.

The *intermedius* type is a slow grower. It forms small, flat, umbonate colonies with a dark grey, slightly raised centre and slightly crenated periphery. It produces no surface growth in broth but gives rise to a fine granular deposit; there is no reversal of pH. Barred forms predominate, about 80 per cent. conforming to this morphology; the rest are granular forms. In fermentative activity it agrees with the *mitis* type; it is not haemolytic.

Not all strains of *C. diphtheriae* conform to these types. A certain proportion of toxigenic strains are not typable and so cannot be assigned to one or other of these types. They are said to be more frequent when diphtheria is mild.

Biochemical Activity. The diphtheria bacillus attacks some of the carbohydrates with the production of acid but not gas. The sugar reactions are studied on Hiss's serum water containing different sugars in the proportion of one per cent. The action on glucose, maltose and saccharose is made use of in the identification of species. The former two are attacked by the diphtheria bacillus but never saccharose (p. 537, Table XVIII). Lactose and mannite are also not attacked. The action of the different types on starch, glycogen and dextrin is given above.

Gelatin is not liquefied Indole is not formed. Litmus milk is unchanged. Nitrates are reduced to nitrites. The *mitis* strains and a few *gravis* strains show haemolytic activity.

Resistance. Most of the bactericidal agents readily destroy this organism. Heat at 58° C. kills it in 10 minutes. Desiccation is not so injurious to it as is the case with many other pathogens. It may remain viable in floor dust for several weeks. Cultures remain viable for several weeks or months. Prolonged cultivation on laboratory media ordinarily impairs the virulence of the organism, but some strains preserve their virulence and toxigenicity even under such conditions. In the dried diphtheritic membrane kept at room temperature and in the dark it retains its vitality and virulence for several months. As mentioned

above, it resists the action of potassium tellurite in certain concentrations.

Types. All strains of *C. diphtheriae* do not behave alike in their biological properties but show a tendency to fall into three more or less distinct groups: *gravis*, *mitis* and *intermedius*. In England the *gravis* and the *intermedius* types are generally associated with severe cases of diphtheria, while the mitis type is usually the one isolated from the milder cases. Such close association with clinical types of diphtheria is not obtained in other countries. For instance, in America the *mitis* type is said to be not infrequently associated with severe or malignant diphtheria. These different types also evince certain distinctness in their colonial appearances when grown on certain differential media like the tellurite blood agar or Clauberg's medium. In their morphological and certain biochemical characters too differences between these varieties are noticeable (p. 523).

C. diphtheriae constitutes an antigenically heterogeneous species. The three types are antigenically distinct from one another. There is also a lack of homogeneity within each of these biological types. The *gravis* strains are divisible mostly into two subtypes, but much is not known about the other two types, except that the *mitis* strains are heterogeneous and that the *intermedius* strains do not show appreciable antigenic differences among themselves. In addition to the type-specific antigen, there is some evidence to show that there is a species-specific antigen, forming a common link between these three varieties.

Not all strains of *C. diphtheriae* are virulent and toxigenic. Avirulent non-toxigenic strains, indistinguishable morphologically and biochemically from the virulent forms, have been described. Such strains may be found in all the three varieties. Their relation to the toxin-producing strains is not clear. Whether they are avirulent variants or avirulent strains sui generis it is difficult to say. Virulent strains may change into avirulent forms, though change in the opposite direction does not occur. On the assumption that only strains that produce the specific exotoxin should be classed as *C. diphtheriae*, some authorities regard that these non-toxigenic strains are *gravis*, *intermedius* and *mitis* types of diphtheroid bacilli, which closely counterfeit the diphtheria bacillus. The available evidence indicates that there is no antigenic difference between the virulent and avirulent strains of

gravis; similar studies have not been made about the *intermedius* and the *mitis* types.

The same kind of toxin is produced by the different types. No qualitative differences in the toxins formed by individual types have been established and the toxin of any one type is neutralisable by the antitoxin produced against any other type. What then constitutes the basis of the difference in the virulence of the types, is by no means clear. Interchange of types has not been observed. It has been suggested that the *gravis* type possesses invasive properties, while the *mitis* type does not. How far this is true or whether it is wholly responsible for the greater clinical severity of the *gravis* type, is not clear. It may be, as has been suggested, that the endotoxin of the *gravis* type also takes part in the pathogenesis.

Toxin Production. *C. diphtheriae* produces a powerful extracellular toxin in fluid media. The same poisonous substance is also formed during natural infection. From the site of infection, where the organism multiplies and forms the toxin, the latter diffuses out into the body through circulation and acting toxically on various organs causes the general symptoms and sequelae of the disease.

Under suitable conditions of growth toxin is produced in abundance. This is by no means the case with all the strains and for this reason the choice of strain for the production of toxin on a large scale is of supreme importance. Many strains refuse to adapt themselves to the artificial culture media and so do not produce in these large quantities of toxin. There does not seem to be any close correlation between the amount of toxin produced and virulence; for, rich toxin-producing strains have been found to cause mild diphtheria. Park-William No. 8, a relatively less virulent strain originally isolated from a mild case, is a rich toxin-producer and is almost universally employed for this purpose.

The production of toxin is markedly influenced by the cultural conditions. A definite alkaline reaction, pH 7·6–8·2, and a free supply of oxygen are very essential for the maximum yield of toxin. The presence of a suitable brand of peptone in adequate amounts (2 per cent.) in an infusion medium was regarded as essential for the optimum production of toxin. But recent studies on the nutritional requirements of the diphtheria bacillus show that a semi-synthetic medium containing, among other things,

iron in certain strength is more favourable for toxin production than the complex infusion-peptone medium. What really matters in the production of a rich yield of potent toxin, appears to be the iron concentration of the medium and not the quality or quantity of peptone present. A medium containing known amino acids, pimelic and nicotinic acids, maltose, calcium and iron yields very satisfactory results. Toxin produced in such a medium is also in a purer state.

For large scale production, Hartley's broth is quite a satisfactory medium. Large conical flasks containing this medium in shallow layers and loosely plugged with cotton are employed. This ensures the supply of plenty of oxygen. These flasks are inoculated with the surface growth from what is called a "starter bottle" in which the strain has been acclimatised to the condition. The organism is allowed to grow in the dark for 7–10 days at 36°–37° C. At the end of this period the bacteria are killed by the addition of an antiseptic, like toluol or phenol, in sufficient strength and removed from the culture by filtration or other means; the crude product thus obtained constitutes the toxin. The so-called toxin, therefore, is not a pure solution of the soluble toxin; in addition it contains other products of bacterial growth and also the ingredients of the culture medium. A potent filtrate may contain as much as 1,000 M.L.D. per cubic centimetre.

Diphtheria toxin possesses all the characteristics of a soluble toxin (q.v.). Its exact chemical composition is not known and it has never been obtained entirely free from the proteins of the medium. Recently, it has been prepared in a relatively pure state by growing the organism in a synthetic medium containing minimal quantities of proteins and isolating the toxin from culture filtrate by salting out with ammonium sulphate followed by dialysis. The refined product is of the nature of a labile protein with a molecular weight of about 70,000 and its M.L.D. for guinea-pigs is 0·0001 mg. (ten million M.L.D. per gram). Diphtheria toxin is unstable and suffers deterioration somewhat rapidly on exposure to air and light. Acids and alkalies, especially the former, destroy it rapidly. Exposure to a temperature of 58°–60° C. inactivates it in one to two hours. If kept in air-tight containers and in the dark, however, the toxin may remain with undiminished potency for many weeks. Evidence of deterioration is the loss of toxic action. It is a powerful poison and is

very toxic to guinea-pigs, in which 0·00025 c.c. may prove fatal within five days. This provides a valuable method for estimating the potency of the toxin. Diphtheria toxin is an excellent antigen and stimulates the production of high potency sera in susceptible animals.

On keeping, the toxin deteriorates rapidly and is converted into toxoid which is atoxic. Though deprived of the toxicity, this modified product, toxoid or anatoxin, is still antigenic and capable of combining with the antitoxin in the usual way. It forms thus a safe immunising agent. The degradation of diphtheria toxin into toxoid can be artificially brought about by the action of heat or chemicals; formalin in 0·3 per cent. strength effects this alteration at 37° C. in two to three weeks. The test of detoxication is that when guinea-pigs are injected with 5 c.c. of the new product subcutaneously or intraperitoneally no symptoms should be produced. The toxoid is extensively used for immunising man against diphtheria and horses in the preparation of antitoxin.

The unit of toxin is the M.L.D. which is the least amount that will, on the average, kill a guinea-pig of 250 g. weight within 96 hours after subcutaneous inoculation. A potent toxic filtrate may contain as much as 1,000 M.L.D. per cubic centimetre. Another unit is the M.R.D. which is the smallest amount which when inoculated intradermally into a guinea-pig will produce a definite reaction. These units are seldom employed in practice for the testing of antitoxin, as a standard cannot be maintained due to the instability of the toxin.

The Production and Standardisation of Diphtheria Antitoxin. A powerful antitoxic serum can be prepared by the repeated injection of horses first with toxoid and then with toxin. The selection of animals and their upkeep during the period of immunisation are important factors. When a high titre has been reached, as shown by assessments of potency conducted after test-bleeding at suitable intervals, the animal is bled through the jugular vein, the serum is seperated, standardised and made available for market. A titre of 600 to 800 units per cubic centimetre is usually obtained after immnisation for two to three months. The antitoxic serum possesses both curative and protective properties.

For purposes of regulating the dose the product has to be standardised. There are three methods available, two of them

based on in vivo and the third on in vitro methods. The biological methods are far more reliable than the in vitro test, as they are based on the protective value of the serum. Of these, Ehrlich's original method is the more reliable and still the official one. The animal chosen is the guinea-pig and several animals are used for the same serum (p. 256).

The smallest amount of antitoxin that will just neutralise 100 M.L.D. of toxin was originally suggested by Ehrlich as the unit of antitoxin. The difficulty is that toxin, on keeping, soon degenerates into toxoid and so cannot be preserved as a stable standard for the standardisation of antitoxin. On the other hand, antitoxic serum is much more stable and, if suitably treated and preserved, keeps for a long time with no appreciable lowering of potency. Ehrlich had also prepared a standard diphtheria antitoxin and preserved it with due care against all possible deterioration. The international unit of antitoxin is based on Ehrlich's original arbitrary standard. "One unit of Diphtheria Antitoxin (1 A.U.) is contained in that amount of antitoxic serum that has the same total combining capacity, for toxin and toxoid together, as one unit of Ehrlich's original antitoxin". This is dried and preserved in vacuo at 0° C. in certain central institutes, and is tested periodically under the auspices of the Biological Standardisation Commission of the League of Nations. It is issued to the licensed producers of commercial serum. All newly prepared antitoxic sera are standardised in terms of this unit.

Ehrlich's method of standardisation is by the subcutaneous injection of mixtures of toxin and antitoxin into guinea-pigs. First, a specimen of fresh toxic filtrate is prepared and its potency assessed in terms of two units. These are the Lo (Limes= Limits) and the L + doses. The Lo dose is the smallest amount of toxin that is completely neutralised by the standard unit of antitoxin. The L + dose is "the smallest amount of diphtheria toxin that, when mixed with one unit of antitoxin and injected subcutaneously into a guinea-pig of 250 g. weight, will, on the average, kill that guinea-pig within ninety-six hours". Having determined these doses, varying quantities of the antitoxic serum under test are separately mixed with L + dose of toxin newly titrated and inoculated subcutaneously into standard guinea pigs. The smallest amount of serum which, when mixed with the L + dose of toxin, will cause a 50 per cent. mortality within

four days in the test animals is taken as containing one unit of antitoxin.

The second in vivo method is based on the observation that diphtheria toxin, when injected into the skin of the guinea-pig, causes certain local reactions. Inoculated intradermally in very small doses (1/250–1/500 M.L.D.) the toxin gives rise to a local swelling and erythema. With slight increase of dose necrosis also supervenes. These reactions can be prevented by a previous neutralisation of toxin with antitoxin. By this method the Lr dose of toxin, *i.e.*, the amount of toxin which, when mixed with a standard unit of antitoxin, produces the minimal skin reaction is found out. Next, varying amounts of the new antitoxic serum are mixed with Lr dose of toxin and 0·2 c.c. of each mixture is injected intracutaneously into a fresh guinea-pig. The amount that causes minimal skin reaction is regarded as containing one unit of antitoxin. The advantage of this method is that a number of toxin-antitoxin mixtures can be tested in one animal.

The third method is based on Ramon's flocculation phenomenon. When toxin and antitoxin are mixed in optimal proportions flocculation occurs. The Lf dose of toxin is first determined by mixing varying quantities of toxin with the standard unit of antitoxin. The amount of toxin contained in the tube that first shows visible flocculation is the Lf unit of toxin. The process is then reversed, varying the amount of serum to be standardised and keeping the toxin constant at the Lf dose. The amount of serum in the tube that first shows visible flocculation contains one Lf dose of antitoxin. Obviously, the Lf dose is a measure of the combining capacity and not of the toxicity only. This is a rough method and is usually employed as a preliminary to the in vivo tests.

The antitoxin is associated with the serum globulin, mostly with the α and β components. The globulin can be precipitated by half-saturation with ammonium sulphate, the precipitate is then extracted with a saturated solution of sodium chloride. The antitoxin can in this way be concentrated and at the same time freed from much of the inert protein material that is responsible for the production of serum sickness. Refined serum is now prepared by treatment of the crude product with proteolytic enzymes, *e.g.* pepsin, which digest the albumin fraction, leaving intact the antitoxin-globulin complex.

Pathogenicity. *C. diphtheriae* is a strict parasite pathogenic for man and experimental animals. The organism, as a rule, does not invade the tissues beyond the primary seat of infection. The powerful exotoxin that is formed and absorbed into the system is mainly responsible for the causation of lesions. Most frequently the pharynx is the primary seat of the infection and the disease called diphtheria is the result. Laryngeal and nasal diphtheria are not infrequent. Other situations that may be affected are the middle ear, conjunctiva and the vulva. Diphtheritic infection of sores is of rare occurrence. Infection of the umbilicus in the newborn has been reported. The bacillus has been isolated from the lungs in a large proportion of cases of fatal diphtheria; whether it is due to a direct infection of this organ, is very doubtful. The isolation of the organism from various internal organs and the blood has also been claimed.

Among the experimental animals, cats, dogs, pigeons and guinea-pigs are highly susceptible. Rabbits are less susceptible than guinea-pigs. Rats and mice are very resistant. Whether the susceptible animal is inoculated with a live culture or with bacteria-free toxin, the result is the same and the animal dies of toxaemia in one to four days, depending upon the dose of the inoculum. The guinea-pig is the usual test animal. Following injection, a soft oedematous swelling appears at the site within twelve to eighteen hours. By this time the animal is obviously ill, showing symptoms of acute toxaemia. In cases where death is delayed beyond the fourth day, the animal may develop acute cachexia and paralysis. Post-mortem examination reveals certain characteristic changes: a grey necrotic area at the site of inoculation surrounded by a zone of congestion; further beyond in the subcutaneous tissue there is marked inflammatory oedema which also involves the deeper structures. The focal lymph glands are enlarged and congested; the liver and the kidneys show degenerative changes; the adrenals are swollen, congested and often haemorrhagic and this is a marked feature; the heart and the diaphragm show fatty degeneration; the lungs are congested and there is effusion into the pleural cavities. If the inoculum is a live culture, then also similar lesions are caused, but the organism, as a rule, remains locally and is not found to invade the tissues, whereas the toxin is readily absorbed into the circulation.

More or less the same changes are observed on post-mortem examination of human cases died of diphtheria; but the adrenal lesion may not be very prominent and only a careful histological examination may disclose it. The most important cause of death in man is the involvement of the heart and the diaphgram.

Diphtheria is an acute infectious disease endemic over wide areas where it frequently assumes epidemic proportions of varying severity. The disease is neither so widely prevalent nor the epidemic so severe in the tropics as in the temperate regions. Limited investigation with the Schick test also reveals the incidence of as high a proportion of Schick-negatives in the tropical countries as in the temperate zone. The reasons for this relative immunity are not clear. Climate may have a part to play. Some workers regard that coloured people are less susceptible to diphtheria; no adequate data are available in support of this. It is doubtful whether such variations are explainable on grounds of any lower virulence of the strains prevalent in the tropics. It is also doubtful whether it can be attributed to any difference in the type incidence of the organism in the tropical countries.

Formerly a disease of rural districts, diphtheria is now endemic in urban areas where it shows a much higher incidence rate than in the rural parts. This is due to the fact that the opportunities for the assemblage of the young susceptible population are far greater in cities and towns than in rural districts.

Overcrowding and congestion favour spread. Due to the same reason diphtheria is a disease of schools, hostels and similar institutions. The close and prolonged contact obtainable in the crowded class rooms and dormitories provides ideal conditions for the rapid transmission of infection; in such places the condition lingers as a smouldering subclinical infection or in the carrier state ready to break out in epidemic form whenever a large number of susceptibles are freshly introduced into that community.

Diphtheria is particularly a childhood disease; it is rare during the first year of life due to immunity passively transferred from the mother. The incidence then steadily rises to a maximum in the age group between two and five years, after which it declines slowly from the fifth to the tenth year and thereafter sharply to a low level. After twenty years the frequency at any age is very low.

The source of infection is the acute case and the carrier. The carrier may have himself suffered from the disease at some

time or other but is still harbouring the infection in his nasopharynx or he may be a simple contact carrier. As in the case of other respiratory infections, the organism is discharged in the secretions of the nose and throat. Spread occurs from man to man by direct contact or indirectly by the droplet method. The organism gains entry through the nose and the nasopharynx which form the common sites of the primary lesion. In a few epidemics milk has been incriminated as the transmitting agent. It is true that diphtheria bacilli can live in milk without producing gross changes. But it seems very doubtful whether milk serves as a common vehicle of infection. Evidence is also against the view that transmission may occur through infected articles, such as toys, drinking cups, pencils, pens and the like. There is some evidence to suggest that infected dust may play a part in transmission.

Carriers. Diphtheria bacilli are found to remain at the site of lesion for varying periods after the disappearance of symptoms. Persons who harbour the infection following recovery from the disease are called convalescent carriers (q.v.). The vast majority of these become free of bacilli in about fifteen days after recovery. Some cases take a longer time for clearance, while a few carry the bacilli persistently. In this connection, it is important to keep in mind that non-virulent non-toxigenic bacilli, resembling in other respects *C. diphtheriae*, may be found in the nasopharynx alone or in association with virulent strains. But, as the avirulent strains never regain virulence, such carriers are not epidemiologically important. Persons who come in contact with clinical diphtheria may also acquire the infection and harbour virulent diphtheria bacilli in their nasopharynx without developing the disease. The organism rapidly disappears from them, but a certain percentage continues as persistent carriers. Contact with the disease is by no means always necessary to establish a carrier state: a few persons who would have never come in contact with diphtheria patients also show the presence of virulent bacilli in their throat; they get the infection from other diphtheria carriers: the condition may be transient or permanent. Those who harbour the bacilli without having suffered from the disease are called healthy carriers or contact carriers. There is no definite data regarding the duration of this carrier state. All such carriers are not necessarily immune; but when immunity exists it must

be due to the presence of antitoxin in their blood. The proof that healthy carriers may also develop immunity comes from the fact that in some of the large cities of the West more than 70 per cent. of the population above six years of age show the presence of circulating antibodies against diphtheria, as indicated by the Schick test. This is not entirely a result of recovery from clinical diphtheria; the carrier state may also account for it. In places where prophylactic immunisation has been extensively practised, the rate of healthy carriers has greatly increased. The carrier rate in a community may, therefore, be an indication of the level of immunity of that community.

Those who harbour infection permanently, either as a complication of the disease or after contact with clinical cases or other carriers, are the chronic carriers. Some pathological condition of the tonsils would seem to be responsible for the persistence of infection in many of these cases, as after tonsillectomy they tend to get rid of the carrier state. The carrier rate varies in different localities and also during different seasons. From published figures an estimate has been made of a 0·6 per cent. healthy carrier rate of virulent diphtheria bacilli among the general non-contact populations of the European countries and a very much higher rate, 7–15 per cent., among contacts. The available evidence tends to show that it may be low in the tropics; no precise information about it is available.

Diphtheria, like tetanus, is essentially a toxaemia. In the natural disease, as in the experimental animals, there is usually no dissemination of the organism into the tissues of the host beyond the immediate neighbourhood of the local lesion. The systemic effects following the absorption of toxin constitute by far the most serious feature. The chief local event is the occurrence of membraneous inflammation. This consists of a toxic degeneration of the epithelial lining followed by a profuse fibrinous exudation from the subjacent tissues, leading to the formation of a pseudo-membrane. This consists of fibrin, dead tissue cells, leucocytes, red cells and bacteria. The process occurs mostly outside the basement membrane and the bacilli are seldom seen far below this. The false membrane is firmly moored down by strands of fibrin, making it difficult to peel. The disease derives its name from the characteristic (leathery) false membrane that so forms. The extent and thickness of the membrane give

an approximate measure of the degree of toxaemia. Hindrance to breathing may be a mechanical consequence of the presence of the membrane. The post-mortem findings are described in a foregoing paragraph.

The *gravis* and the *intermedius* types are responsible for the severer forms of the disease in many localities; the *mitis* is relatively mild. This is not a universal observation and variations may occur in different places and in different epidemics (p. 525). It is a well attested fact that the mortality rate has been considerably reduced after the introduction of serum therapy. The mortality rate is highest in the *gravis* type of infection and least in the *mitis* type. The figures from several epidemics show an average of about 10 per cent. fatality rate. The success of antitoxin treatment is most noticeable in the *mitis* and least in the *gravis* type of infection.

Immunity. Immunity to diphtheria is essentially an antitoxic immunity. The immunity developed as the result of inapparent infection or after prophylactic immunisation is also of the same nature. Recovery from the disease confers a high degree of immunity and is associated with the presence of antitoxin in the blood; second attacks are very uncommon.

Diagnosis. The clinician should not wait for a bacteriological confirmation of diphtheria. As soon as he suspects a case, he should administer the specific treatment and take the necessary precautions. However, the bacteriological examination should never be omitted. At the same time it should be remembered that the isolation of the diphtheria bacillus from the throat is not always a proof that the disease is diphtheria, as much as a failure to do so is not always a proof against the disease.

By direct microscopy a presumptive diagnosis is made in acute cases. It has some value and, where conditions permit, should be followed as a routine. The inflammatory exudate from the site of the lesion is taken by means of a sterile swab, carefully rubbed over the false membrane or the mucosa over the affected area. Smears are made from the material; one is stained by the Gram's method and another by any of the special stains—Loeffler's methylene blue, Neisser's stain or Pugh's stain. In acute cases the characteristic weakly Gram-positive bacilli are usually found in large numbers. A positive finding, however, depends not a little on the way in which the material is taken.

A point to remember in connection with smear examination is that, as most of the *gravis* strains lack the characteristic metachromatic granules, their identification may present real difficulty. Again, morphologically identical, but avirulent, organisms are sometimes found in non-diphtheritic, and even in normal, conditions of the nasopharynx. Again, the condition may be an independent throat affection in a carrier with a virulent toxigenic strain. A negative finding is also not of much value. The presence of Vincent's organisms in the absence of diphtheria bacilli has some indirect diagnostic value, although it does not altogether negative diphtheria. Double infections with diphtheria and Vincent's organisms are by no means rare.

It is not safe to place much reliance on direct examination; isolation of the organism is essential and for this purpose the material is inoculated on a Loeffler's serum slope as well as on a tellurite plate. Diphtheria bacilli develop earlier on Loeffler's than on tellurite medium, enabling, in some cases at least, an earlier diagnosis. Occasionally tellurite-sensitive strains of *C. diphtheriae* are encountered; consequently, in the absence of Loeffler's culture a false negative may be the result. The inclusion of Loeffler, therefore, ensures greater safety. Further, it is advantageous to inoculate as a routine a blood agar plate as well, as it enables the detection of haemolytic streptococci. After eighteen to twenty-four hours the cultures are examined and if Loeffler and tellurite are both positive an immediate report is sent. It will be recalled that the characteristic morphological features are not developed satisfactorily on tellurite medium. When stained smears show the presence of the characteristic bacilli on Loeffler's slant, while the tellurite plate remains negative, a provisional diagnosis is sent. But it should be kept in mind that occasionally growth on tellurite is delayed up to forty-eight hours. The tellurite medium is valuable not only as a selective medium for isolating the organism but also for determining the type; as discussed above, under certain conditions the colonial appearances on this medium are type-characteristic. But certain other tellurite-reducing organisms, like staphylococci and micrococci, often found in the nasopharynx also produce colonies resembling those of the *mitis* type, but they are blacker. Also, a Loeffler-negative may on rare occasions turn out to be tellurite-positive, so that tellurite gives a higher proportion of positive results than

Loeffler. In such cases proper colonies from the tellurite plate should be subcultured on Loeffler's serum slopes and the growth examined microscopically, because, as noted above, microscopy of tellurite colonies is not characteristic. By these procedures alone a fairly correct diagnosis can be arrived at in a high proportion of cases of throat diphtheria. But in nasal diphtheria or in the diphtheritic affection of other sites and wounds and when dealing with diphtheria carriers, it is necessary to proceed with further identification tests.

On morphological grounds the differentiation of the diphtheria bacillus from Hofmann's bacilllus may often give some difficulty, especially in *mitis* cases. Hofmann's bacilli are strongly Gram-positive, relatively stout and short, usually non-granular but presenting often a diplococcal appearance due to the presence of an unstained bar in the middle of the organism.

After isolation the study of the biochemical and haemolytic properties should be proceeded with. The following table gives the sugar reactions. But these are only of subsidiary importance. For, in spite of agreement on all these points, there is no certainty that the strain isolated is toxigenic and virulent. This can only be decided by the virulence or pathogenicity test.

TABLE XVIII

Biochemical Reactions of C. Diphtheriae and Certain Allied Species

	Glucose	Maltose	Saccharose
C. hofmannii ..	−	−	−
C. diphtheriae ..	+	+	−
C. acnes ..	+	+	±
C. xerose ..	+	+	+

Virulence test should always be done in the case of bacilli isolated from all forms of the disease, except the acute throat type, and from carriers. The guinea-pig is the animal of choice. Rabbits may also be employed. *C. diphtheriae* never ferments saccharose and by a preliminary fermentation test such strains as ferment this sugar are eliminated. A pure twenty-four hours' growth on Loeffler's serum slant, emulsified in sterile broth (saline

has been found to destroy the organism) and standardised to contain about 500 million bacilli per ml. forms the test material. The test may be performed either by the subcutaneous or by the intradermal method of Romer; the latter obtains greater vogue, as it does not involve the sacrifice of animals and as it allows the testing of several strains on the same animal. In this, two young healthy white guinea-pigs, each weighing 400 g., are chosen, one as the test animal and the other as the control. The hair from the abdomen is removed either by shaving or by the application of a depilatory. The control animal receives overnight 500 units of antitoxin intraperitoneally. The following morning the parts are cleaned and 0·2 ml. of the suspension is inoculated intradermally into each animal and the site of injection marked by a coloured pencil. Thus, several cultures can be tested simultaneously on the same animal. If more than three or four specimens are injected at the same time, the test animal also should be given 100 units of antitoxin four hours after the injection of cultures so that it may not die of the cumulative effect of the inoculum. A positive test is indicated by the production of a definite local inflammatory lesion, about 15 mm. in diameter, at the site of injection after twenty-four hours followed by superficial necrosis in three to four days and later healing, while the control pig shows no such reaction. Absence of reaction in both the test and control animals is evidence of non-virulence of the suspected strain.

In the subcutaneous method 0·5 c.c. of the saline suspension is injected subcutaneously into each of two guinea-pigs, one of which has previously received a protective dose of antitoxin. While the control animal remains alive, the test animal dies in twenty-four to ninety-six hours, presenting characteristic post-mortem appearances, if the organism is virulent.

Prophylaxis. The prevention of diphtheria is a major public health problem in many countries. Active immunisation is the chief specific measure employed in combating the spread of infection. Wholesale immunisation of a community is neither practicable nor necessary; it is only the susceptibles that need such protection and they are mostly children below the age of eight years. Infants under six months are not usually immunised. The most suitable time for active immunisation is between one and five years. The detection of the susceptibles is obviously an

important preliminary step and is done by means of the Schick test. In actual practice, however, Schick testing as a routine measure preliminary to immunisation is not always feasible; children under eight years of age are generally non-immune and the test may, therefore, be omitted below this age.

Schick Test. This test actually makes it possible to discover whether or not diphtheria antitoxin is present in the circulating blood of an individual in sufficient quantity to ward off an attack of diphtheria. It is done by injecting intradermally a minute quantity of diphtheria toxin which is an irritant to the normal tissue. A positive test shows either that the person has no antitoxin in his blood or, if he has, the titre is less than 1 : 100 to 1 : 200 A.U. per c.c., which is generally inadequate to protect him against an attack. With this or a higher concentration of antitoxin in the blood the injected toxin is neutralised in the tissues preventing any local reaction; the test is then negative. It may be noted that the antitoxin content among the Schick-negatives may vary widely and that no rigid Schick-negative level can be laid down. The positive reactor is the susceptible and he should be protected, whereas the negative reactor either does not get the disease at all or, if he does, only the mildest form. There is also the possibility that diphtheria occurring in the Schick-negative individuals may be the *gravis* type. It is seldom fatal.

The test is done by injecting intracutaneously on the anterior aspect of the forearm one-fiftieth of a minimum lethal dose of diphtheria toxin contained in 0·2 ml. of the inoculum. This amount is the original Schick test dose; it contains also some toxoid; the dose is now so adjusted that it should be just enough to neutralise a thousandth part of an international unit of antitoxin. The employment of a larger dose will produce reaction even in those individuals who have a sufficiency of antitoxin in their blood for protection. As a control, an equal quantity of the same toxin, detoxicated by heating to 70° C. for 5 minutes, is injected on the other arm. The arms are examined every day and the result is read on the fourth and seventh days; if only one reading is possible, the seventh day is to be preferred. A negative reaction is indicated by the absence of any change in either arm. In a positive reaction, while there is no change in the control arm, the test arm shows the appearance of a red inflammatory swelling at the site in twenty-four hours; this

increases to a maximum size in four to seven days when it should be 1–2 c.m. or more in diameter; thereafter it gradually disappears in a few days, leaving a brownish pigmentation and slight desquamation. In the positive case the toxin irritating the tissue gives rise to the local inflammatory reaction, whereas in a negative case the toxin is neutralised and rendered innocuous to the tissues by the antitoxin present in the tissue fluids (see above). A pseudo-reaction is occasionally seen. It is allergic in type and is caused by other protein constituents of the inoculum. It may also be the result of sensitisation with diphtheria bacilli. A slight flush is produced on both arms in twenty-four hours. It is not sharply defined and subsides rapidly, leaving little or no evidence by the fourth day. A combined, pseudo and positive, reaction is marked by a slight reaction on the control arm and a more exaggerated reaction on the test arm. By the fourth day the latter becomes unmistakable while the reaction on the control arm would have practically vanished.

As the immunity in diphtheria is predominantly antitoxic in nature, prophylactic immunisation aims at the creation of this type of immunity in the susceptible individuals of a community. The material used for vaccination, therefore, is the toxin or its products. Several preparations are available. The employment of the toxin is too dangerous for human beings; even a very minute dose will cause intense local and possibly systemic reaction. Hence, it is never used for the immunisation of man.

The Toxin-Antitoxin Mixture. This is now seldom used, as it is not free from risk; the introduction of the toxoid, a highly antigenic but relatively harmless and so safe product, has also contributed to its disuse. Each c.c. of the mixture usually employed contains 0·1 L+ dose of toxin and 0·08 unit of antitoxin; the toxin is therefore slightly under-neutralised. It is liable to produce severe reactions. Rarely it causes serious accidents due to the presence of excess of free toxin from several causes, such as the development of Danysz Phenomenon (p. 255) if adequate attention is not paid on the mode of mixing, dissociation of toxin and antitoxin under the effect of freezing during preservation or inactivation of antitoxin by the preservative antiseptic. Three doses of 1 c.c. each, administered subcutaneously at intervals of one to two weeks, form a course.

Formol-Toxoid (F.T.). It is the detoxicated product derived by the treatment of toxin with formalin (p. 211). The toxoid is a powerful antigen with a greater immunising capacity than the toxin-antitoxin mixture. It is free from any serum protein, but older children and adults often show marked sensitiveness to it because of the presence of protein from the culture media. Hence, it is not advocated for individuals over the age of ten years. The sensitiveness can be elicited by the Molony test which is done by the intradermal injection of 0·2 c.c. of a weak dilution of the toxoid, 1 : 100. The appearance of a local erythema and induration in the course of forty-eight hours constitutes a positive reaction. In the absence of a positive reaction formol-toxoid can be given with safety; it is administered intramuscularly, the usual course consists of three doses of 0·5, 1·0, and 1·0 c.c. at intervals of two to three weeks.

Toxoid-Antitoxin Mixture (T.A.M.). This reagent is a partially neutralised toxoid and is less likely to cause reactions than formol-toxoid. But it is a less potent antigen than the latter. Three injections of 1 c.c. each are given intramuscularly at intervals of two to four weeks. It is generally sufficient to confer adequate protection.

Toxoid-Antitoxin Floccules (T.A.F.). This is a saline suspension of the precipitate formed when toxoid is neutralised with an equivalent amount of antitoxin. It has a high antigenic value and causes but a slight reaction. The dosage is the same as that of T.A.M.

Alum-Precipitated Toxoid (A.P.T.). The addition of a small amount of alum to formol-toxoid causes the precipitation of the latter. The precipitate, after being washed, is resuspended in physiological saline; it is relatively insoluble, forming a turbid suspension. The A.P.T. is also refined as most of the culture proteins in the original toxoid is discarded in the supernatant fluid. It possesses a high immunising value. Even a single dose of 0·5 c.c. produces excellent results; but it is advantageous to give two doses of 0·5 c.c. each at an interval of four weeks. With such a course about 98 per cent. of Schick-positives are converted into Schick-negatives, whereas the conversion rates of other prophylactics are always less than this. The advantage of A.P.T. is its prolonged stimulant action due to its insolubility and consequent slow release of the toxoid from the site of injection.

The diphtheria prophylactics are standardised in accordance with the rules laid down in the Therapeutic Substances Act.

The development of immunity is a gradual one, occupying at least three months. A Schick test may be conducted on the immunised subjects two to three months after the last injection to confirm the results of immunisation. The immunity developed may last for five to six years. It has been advocated to reimmunise children every four or five years up to the age of fifteen. For this purpose one single dose is enough; it is followed by a brisk rise in the antitoxin titre of blood.

Prophylactic Use of Antitoxic Serum. Passive immunisation by the administration of antitoxic serum has only limited application. It is not practical to control the spread of diphtheria by this method. Now it is resorted to only as an emergency measure. Delicate children or hospital attendants who are exposed to infection may be protected by the injection of 500–1,000 units of antitoxin. The effect does not last longer than two or three weeks.

The combination of active and passive immunisation, in which both toxoid and antitoxin are simultaneously injected into opposite arms, has been advocated in certain circumstances. Under the transitory protection of passive immunity, active immunity develops. The passive protection does not seriously interfere with the process of active immunisation.

Treatment. The specific treatment of diphtheria consists in the administration of antitoxin which will neutralise the circulating toxin. Since its value as a therapeutic agent was first demonstrated by Behring and Kitasato in 1890, its usefulness has been abundantly established both by experimental and clinical investigations. The mortality rate and the occurrence of complications have both been markedly reduced by serum therapy.

Where symptoms suggest diphtheria, the specific serum must be immediately administered without waiting for the bacteriological confirmation. Even a provisional bacteriological report cannot be sent in less than eighteen to twenty-four hours. The greatest benefit is derived by giving serum early in the disease, but it should not be withheld even if the case is seen in the advanced stage. With each day's delay the fatality rate progressively increases. The dose must be adequate and to err on the excess side is safer than giving too little. Larger doses are indicated in

older patients and in severer cases. Under the age of two years, 2,000 to 10,000 units may be given, depending upon the severity of the disease; between two and fifteen years 3,000 to 20,000 and in adults up to 50,000 units may be given. Given by the intramuscular route, the serum is absorbed into the circulation sufficiently rapidly, but, where quicker action is desired, the intravenous route must be chosen.

When the disease is due to the *gravis* type serum treatment scarcely confers any benefit and the mortality rate remains high. The reason for this discrepancy is still obscure. No immunological difference has been made out beyond doubt between the toxins produced by the different types of *C. diphtheriae*. Several explanations have been offered to account for this mysterious behaviour of the *gravis* type towards antiserum. The rate of toxin production in the *gravis* type may be too high to be neutralised as quickly as it is formed or the avidity of the antitoxin for the toxin of the *gravis* type may be low. It may be that, in addition to the toxin, other toxic factors, such as the endotoxin, may also be contributing to the greater severity of the *gravis* type.

Corynebacterium Hofmannii. Hofmann's bacillus is a diphtheroid found as a commensal in the throat.

Morphology and Staining. It is shorter than the diphtheria bacillus and often presents a bulged out oval appearance. It is non-motile. In stained preparations an unstained central septum gives it a diplococcal appearance. Unlike the diphtheria bacillus, it is strongly Gram-positive and usually devoid of metachromatic granules.

Culture. The growth is similar in character to that of *C. diphtheriae* but more abundant and the colonies are larger and more opaque. Growth is not inhibited by the presence of tellurite.

Biochemical Reactions. It is inert. *Vide* table on page 537.

Toxin Production and Pathogenicity. In both these respects Hofmann's bacillus is inactive.

Corynebacterium Xerose. The xerosis bacillus is another diphtheroid; it is a normal inhabitant of the conjunctival sac.

Morphology and Staining. This organism bears a close resemblance to the diphtheria bacillus, but a barred appearance is the predominating feature and metachromatic granules are infrequent. It is non-motile.

Culture. The colonies are smaller than those of *C. diphtheriae*. Some strains are slow growers. Tellurite does not inhibit its growth.

Biochemical Reactions. Refer to the table on page 537. The xerosis bacillus ferments saccharose, a point of difference from *C. diphtheriae*.

Toxin Production and Pathogenicity. No toxin is produced by the xerosis bacillus; no pathogenicity has been established for it.

Corynebacterium Acnes. This organism is found in acne lesions in association with a white staphylococcus and is regarded as aetiologically related to acne.

Morphology and Staining. It is a diphtheroid, usually measuring about $1 \cdot 5 \times 0 \cdot 5$ micron and markedly pleomorphic. It is non-motile, weakly Gram-positive and sometimes beaded.

Culture. The acne bacillus is a microaerophilic organism. Growth occurs aerobically in a medium containing blood or serum with a pH ranging between 6·2 and 6·8. Much better growth is obtained under anaerobic conditions and for primary isolation such methods have to be employed. Glucose improves growth and in a glucose agar shake culture fairly large discrete colonies are formed at the bottom of the tube. Older colonies on solid media have a rose-coloured tint.

Biochemical Reactions. Refer to the table on page 537.

Toxin Production and Pathogenicity. Its constant presence in the pustules of acne vulgaris suggests an aetiological relationship.

CHAPTER XXVIII

MYCOBACTERIUM

The organisms comprising the genus *Mycobacterium* are slender rods which are stained with difficulty, but when once stained they are acid-fast, *i.e.*, they resist decolorisation with mineral acids. The cells sometimes show swollen, irregular and branched forms. Growth is slow for most species. They are non-motile, non-sporing, Gram-positive and aerobic. Several species are pathogenic to man and animals.

The first member to be described was the leprosy bacillus or *Mycobacterium leprae* by Hansen in 1874. In 1882 Koch discovered the tubercle bacillus or *Mycobacterium tuberculosis*; he isolated and grew the organism in pure culture and brought forward evidence to prove its causal relationship to tuberculosis. Subsequent work established the existence of two types of *Mycobacterium tuberculosis*: the human and the bovine, *Mycobacterium tuberculosis var. hominis* and *Mycobacterium tuberculosis var. bovis* respectively. Koch's work was followed by the discovery of the avian type, *Mycobacterium avium*, by Rivolta (1889) and of the "cold-blooded" types, causing disease in the birds and the cold-blooded animals respectively. Subsequently, various saprophytic species were discovered: *Mycobacterium smegmatis* or the smegma bacillus frequently present in the smegma of both sexes, *Mycobacterium phlei* or the timothy-grass bacillus, *Mycobacterium stercoris* or the mist (manure) bacillus found in cattle manure, *Mycobacterium butyricum* or the butter bacillus and several others. Another acid-fast bacillus is *Mycobacterium paratuberculosis* or Johne's bacillus described by Johne and Frothingham (1895) from cases of chronic enteritis in cattle, now known as Johne's disease. Stefansky (1903) found an acid-fast organism in the lesions of rat leprosy, *Mycobacterium leprae murium*. Wells (1937) isolated an acid-fast bacillus from naturally occurring tuberculosis-like lesions in voles. For it the name *Mycobacterium muris* has been proposed.

Mycobacterium Tuberculosis. This organism is the cause of tuberculosis in man and cattle. The species is divisible into two varieties, the human and the bovine; the disease they cause is sometimes referred to as mammalian tuberculosis to distinguish it from tuberculosis of birds and of cold-blooded animals. Now the recently discovered vole tuberculosis has also to be included in the mammalian form.

Habitat. The tubercle bacilli are strict parasites and do not multiply outside the host tissues. But they are found to remain alive and virulent for considerable periods in dust, towels, books and other contaminated articles, their source in these being mainly the sputum, nasal and oral discharges and sprays, urine, etc., containing the organism.

Morphology and Staining. They are slender rods, 0·5—4·0 by 0·3 to 0·6 micron, straight or slightly curved with rounded ends, arranged singly or in small clumps, staining uniformly or irregularly. In culture they are shorter and more slender than in tissues. Club-bed, filamentous and branching forms are rare features seen when growing in artificial media; branching forms are more frequent in cultures of the avian type. The occurrence of true branching and filamentous forms shows the close taxonomic relationship these bacilli bear to the higher fungi, hence the name *Mycobacterium*. Bovine strains are said to be shorter, stouter, straighter and less granular in appearance than the human strains, but, as the morphology of both is variable, it does not always help in their identification. The tubercle bacilli do not form flagella, spores or capsule. They have an envelope with a high content of lipoid.

The tubercle bacillus is very resistant to staining by the ordinary methods. Hence, several special methods have been devised, all based on the property of acid-fastness. A strong dye, such as the basic fuchsin, is employed with phenol as a mordant. Even then prolonged staining or the application of heat is necessary. Once stained, the organism resists subsequent decolorisation with dilute mineral acids. The best and the most widely used one is the Ziehl-Neelsen method. Stained by this method, the acid-fast bacilli appear red on a blue background, the tissue cells and other organisms also taking a blue tinge. Some workers prefer a yellow counterstain and use picric acid, one per cent. Bismarck brown or some other stain. Heating

helps to soften the waxy material which is thus rendered more permeable to the aqueous dyes, and phenol serves as a mordant. The property of acid-fastness appears to be due to the presence of an unsaponifiable chloroform-soluble wax in the envelope of the bacillus. The greater the amount of this material present the greater is the resistance to decolorisation. The tubercle bacillus has been shown to contain more of this wax and is, therefore, more acid-fast than any of the other acid-fast organisms. This explanation of acid-fastness is not universally accepted.

The tubercle bacillus is also alcohol-fast and Gram-positive, but considerable difficulty is experienced in staining it by the ordinary Gram's technique. Hence, it is not employed as a routine procedure in the examination of materials. There is no difference in the staining property between the human and the bovine types.

Granular and filterable forms of *Myco. tuberculosis* have been described (p. 333). The former are called Much's granules after the first describer; they are non-acid-fast and require the special staining method devised by Much to show them up. Others also have seen these forms. They are said to be virulent and capable of growing into the typical acid-fast rods. It may be realised that large numbers of acid-fast bacilli, perhaps 100,000 or more per cubic centimetre, should be present in the specimen to enable a positive diagnosis to be made by smear examination. Hence, when a culture is grown from a microscopically negative material, it may be from missed bacilli and not necessarily from any granular variants. The question, therefore, should be kept subjudice, pending further studies. Many workers consider the granules as degeneration products. Much has also described the finding of non-acid-fast bacillary forms in the lesions. Such forms may occur in young cultures. The occurrence of filterable forms is still more doubtful. The granular and filterable forms have been interpreted by the upholders of their occurrence as indicative of a regular succession of morphological states, or cyclogeny, which the tubercle bacillus goes through. The evidence so far as it goes does not support this assumption (p. 342).

Growth Requirements. The tubercle bacillus is an obligate aerobe, refusing to grow under strict anaerobic conditions. A high oxygen tension and plenty of moisture are essential for its growth. Plenty of condensation water, therefore, makes the

medium more favourable. In sealed tubes the organism ceases to grow after three or four weeks. The optimum temperature is 37° C. for the mammalian types, as compared to 40° C. for the avian and 25° C. for the cold-blooded forms. No growth occurs below 30° or above 42° C. in the case of the mammalian types, but the cold-blooded and the saprophytic strains have a much wider range of temperature.

Mycobacterium tuberculosis requires special culture media for growth. Even on these it grows very slowly and takes a week or more to grow into macroscopic size and four to six weeks to give an abundant growth. The human type grows luxuriantly, particularly when the medium is glycerinated, but the growth of the bovine type is relatively poor. For this reason, the former is termed *eugonic* and the latter *dysgonic*. The most satisfactory media are the insipissated serum, coagulated egg and glycerinated potato. Certain more complex media, like Petroff's, Lowenstein's and Corper's, have been claimed to be superior to these. Though growth does not occur in ordinary media, like nutrient agar and broth, the addition of 5–6 per cent. glycerol renders them suitable for the growth of all types. But this suitability is more apparent in the case of secondary cultures and reliance should not be placed on this method for primary isolation. The human type grows more profusely on the special media when glycerol is added, whereas the growth of the bovine type is not influenced by this. Some observers consider that glycerol may even inhibit the growth of the bovine type. Glucose also acts more or less in the same way as glycerol.

Several synthetic media have been devised for growing the tubercle bacillus; one of the best known is Long's synthetic medium which contains glycerol, asparagin, citrate and inorganic salts: the tubercle bacilli are best grown on this when they are required for the study of their chemical constitution.

Cultural Characters. The character of the growth depends upon the type of the organism and the medium employed. On coagulated beef serum the human type presents a thin, effuse, confluent, finely granular, ground glass appearance with a grey and later yellow colour. The bovine type shows the same growth character on this medium but does not produce pigmentation. On coagulated egg the growth of the human type is dry discrete or confluent, slightly raised with a coarsely granular, nodular

or heaped-up appearance and a grey or buff to yellow colour. The bovine type shows poorer growth and no pigmentation. A fruity odour is a peculiar feature of the cultures of the tubercle bacillus. Another striking feature of its growth is its tough and tenacious consistency. Though friable, the growth is emulsified with great difficulty. A uniform emulsion can be made only by grinding up the growth for a long time. In glycerine broth the tubercle bacillus does not produce any turbidity but grows in a thick, white or cream-coloured and corrugated or wrinkled pellicle, extending up the sides of the flask. In course of time a granular or scaly deposit is also formed at the bottom. Bovine strains give rise to a thin, greyish white film with a slightly nodular appearance; a slight granular deposit is produced but no turbidity. The avian type grows more freely on all media than the mammalian types and the cold-blooded ones more readily than the avian. The saprophytes are easy growers.

Biochemical Activity. Very little is known about the biochemical activities of this organism. The tubercle bacilli are able to grow in milk, but no visible change is produced. Some of the sugars, like glucose, arabinose and sometimes saccharose, are attacked with the production of acid without gas; lactose is not attacked. There is no evidence of indole production. The bovine variety renders glycerol broth alkaline, while the human type makes it slightly acid.

Glycerol is readily utilised by the human type, but this activity is very much restricted in the case of the bovine type. The human type develops an yellowish to red pigment, while the bovine variety never produces pigment.

Resistance. The tubercle bacillus is destroyed by heat very rapidly. Boiling milk for one minute kills it. Sunlight and ultraviolet rays are also rapidly lethal, the culture form being killed within two hours of exposure to the former. But the organism resists desiccation for a long time. It is also relatively resistant to chemical agents and use is made of this property in devising means for the isolation of the organism. Marked resistance is evinced to 5 per cent. phenol, antiformin, 4 per cent. sodium hydroxide or 15 per cent. sulphuric acid, all of which kill off the associated organisms in five to twenty minutes, but do not interfere with the vitality of the tubercle bacillus. A 5 per cent. phenol takes about 24 hours to destrov the organism.

Hypochlorites are almost devoid of any lethal effect on it. Some of the coal-tar dyes in certain dilutions do not affect the growth of the tubercle bacillus, while in the same dilutions they suppress the growth of many other contaminating organisms. This fact is taken advantage of in the preparation of several selective media, *e.g.*, gentian violet is incorporated in Petroff's medium, crystal violet in Corper's medium and malachite green in Lowenstein-Jensen's medium. The organism can survive long, for weeks or months, in putrefying sputum or other materials. Minute particles of sputum, dried so completely as to float about in the air as dust, may be infective up to ten days. Similarly, if the sputum is dried and preserved in a cool dark place, the organism maintains its viability for as long as six to eight months. The gastric juice does not form an efficient barrier to its passage through the stomach. Perhaps the high content of wax is what imparts the high degree of resistance to the tubercle bacillus.

Chemical Structure. The chemical constitution of no other micro-organism has been so closely studied as that of the mycobacteria. The tubercle bacilli show a relatively high content of lipoidal materials which may be up to 41 per cent. of the dry weight. Proteins form about 50 per cent. of the dry weight and they are mostly composed of nucleoproteins. The proportion of polysaccharides is very low; they may be of the nature of haptens. The relative amount of fat in the various acid-fast-bacilli varies considerably; it is highest in the human type of tubercle bacilli and lowest in the saprophytic types. The reverse seems to be the case with the polysaccharide content.

The lipoids are made up of neutral fats or glycerides, phosphatides and wax. Phospholipoids contain several saturated and unsaturated fatty acids. Besides palmetic, linoleic and linolenic acids, there are two others which are specific to the tubercle bacilli. They are phthioic acid, isomeric with cerotic acid and optically active, and tuberculo-stearic acid, isomeric with stearic acid and optically inactive. The waxy substance is also complex in structure and contains a high percentage of unsaponifiable fraction (mykol) which is composed of higher alcohols and responsible for the acid-fast reaction. From the neutral fat of the human tubercle bacillus a yellow pigment, phthiocol, has been isolated. The proteins of the tubercle bacilli contain different complex components, of which at least two appear to be antigenic.

Differences seem to exist between the proteins of the saprophytic and pathogenic types and even among the various species of the latter.

Phthioic acid, when introduced into experimental animals, induces the multiplication of epithelioid cells, and the unsaponifiable acid-fast wax that of undifferentiated connective tissue cells. The tuberculo-proteins cause tissue allergy and seem to stimulate the formation of monocytes, epithelioid cells and giant cells in tubercles. They constitute the active agents in the tuberculins. The significance of polysaccharides has not been well studied. They may play a part in the pathogenesis probably by neutralising the opsonins and also by exerting a toxic action on the neutrophil leucocytes.

Antigenic Structure. The antigenic structure of the tubercle bacilli is not fully worked out. By serological methods they are classifiable into three distinct groups: the mammalian, the avian and the cold-blooded. It is impossible to distinguish the human from the bovine variety by serological means and they seem to constitute a homogeneous group. However, they can be separated by their cultural reactions and pathogenicity tests. Tuberculins prepared from these two types are indistinguishable in their action. The recently discovered murine type does not seem to differ serologically from the other mammalian types. Existing evidence suggests that the human, bovine and avian types share a common antigen, but the avian has also an antigen not found in the other two types. What confers group specificity to *Myco-tuberculosis*, may be a common polysaccharide, while the proteins may be responsible for type specificity.

Variation. Although extensive studies on the subject of the variability of *Myco. tuberculosis* have been conducted of late, the results achieved so far are in no way conclusive. Certain variant forms have been described as occurring spontaneously in culture. Such are the granular and filterable forms discussed in a previous section. Several workers have reported the occurrence of S–R variation with associated degradation of virulence, but this seems very doubtful and evidence in support of it rests more on subjective interpretation than on objective proofs. It is true that sometimes on certain culture media *Myco. tuberculosis* gives rise to soft colonies which are butyrous in consistence and readily emulsifiable in contrast to the usual rough rugged type

of colonies which are very difficult to emulsify. But such colonies have none of the qualities characteristic of the S–R variation of other bacteria. On the other hand, the evidence is that they are environmental, promptly returning to the common form on transplantation to the usual medium. The artificial production of an avirulent variant, the B.C.G., is described in a later section.

Toxin Production. No diffusible toxin is developed by the tubercle bacillus and the active principle is the endotoxin liberated by the disintegrating cells. The endotoxins of the different types are closely related. Tuberculin, even in fairly large doses, is not toxic to the non-tuberculised subject.

Pathogenicity. The mammalian and the avian types are strict parasites. The human variety causes spontaneous disease in man, monkeys, pigs, dogs and parrots; the bovine type in cattle, pigs, horses and man and the avian type in birds and occasionally in pigs, sheep and cattle. The cattle tuberculosis is almost exclusively due to the bovine type. Fowls and other domestic birds are insusceptible to the mammalian forms.

Experimentally all laboratory animals, especially guinea-pigs and rabbits, are very susceptible to the mammalian types and relatively less so to the avian type. The bovine type is more virulent to the bovines and the laboratory animals than the human type. In the cattle the former produces a fatal tuberculosis, whereas the latter produces only a localised lesion which soon heals. Injected into the rabbit intravenously (0·01 to 0·1 mg. of dried bacilli) or subcutaneously (10 mg.), the bovine variety causes generalised tuberculosis and death in 6–12 weeks, whereas the human type, in identical dose and given intravenously, causes only localised lesions confined to the lungs and kidneys, death either not ensuing or ensuing only after a longer interval. If the human type is given subcutaneously, the lesion is confined to the spot and shows no tendency to spread. The cattle type is also more virulent to guinea-pigs than the human type; but both kill them in six to fifteen weeks, the former doing it much earlier than the latter. The human strains are thus more virulent to the guinea-pig than to the rabbit. Recently, voles have been found to be highly susceptible to the bovine strains, while they are resistant to the human strains.

The avian type is very much less virulent to the guinea-pig and rabbit than the mammalian types. Of these, the guinea-pig

is less susceptible than the rabbit. Subcutaneous inoculation into the former produces only a localised abscess with no visible tubercle formation. Large intraperitoneal doses prove fatal in a few weeks. On post-mortem examination no macroscopic tubercles are visible, but cultures and smears from the liver and spleen show the presence of the organism. The disease in which there is multiplication of the bacilli without macroscopic tubercle formation is called the *Yersin type* of tuberculosis.

Whether injected with tuberculous material or culture of either type, guinea-pigs die of generalised tuberculosis in six to fifteen weeks; rarely death may be delayed even up to a year or more. Following subcutaneous inoculation, usually into the left thigh, a local swelling appears which later caseates and ulcerates, discharging caseous matter containing innumerable bacilli. If the injection is given intramuscularly, ulceration does not occur and for this reason this method is more advantageous. The focal lymphatic glands are soon affected and the infection then extends to other lymphatic glands of the body. Dissemination of infection soon follows and the animal dies of generalised tuberculosis. Autopsy shows a caseous local lesion; enlarged and caseous focal glands, the caseation becoming less and less marked with the distance of the glands from the primary lesion; the spleen is several times enlarged and mottled with yellowish white, irregular areas of necrosis, varying greatly in size; the liver is enlarged, presenting a similar but less marked appearance; the lungs show relatively slight lesions and the kidneys practically none. The necrotic foci on an enlarged spleen and liver are the most arresting features in the gross pathology; they are peculiar to the guinea-pig. *Pasturella pseudotuberculosis* causes a spontaneous disease in guinea-pigs, pseudotuberculosis, with lesions somewhat identical to those of tuberculosis. This must be carefully excluded by microscopical and cultural examination.

Whatever form tuberculosis may take, the underlying histological changes are usually the same and consist of the formation of the *tubercle* or *miliary tubercle*. It is the constant presence of these tiny nodules in all advanced lesions caused by the tubercle bacilli that gives the disease its name. Early tubercles are minute greyish white, translucent nodules about the size of a millet and plainly visible to the naked eye and having a definite histological structure. Generally, they have their origin mainly from the

local tissues. The fixed connective tissue cells surrounding the bacillary invader proliferate forming what are called *epithelioid* cells (epithelial-like) with large vesicular nuclei. Several layers of these cells are thus formed. They form the essential feature of the histology of the tubercle. The stimulus for their formation is probably partly chemical due to certain of the chemical components of the disintegrating bacilli (p. 551) and partly mechanical due to the presence of the organism. Giant cells (Langhan's type) soon make their appearance in the developing tubercle. They are large multinucleate masses of protoplam with a distinctive appearance. Their precise origin is by no means established. Many hold that they result from the fusion of several macrophages (epithelioid cells), forming large plasmodial masses containing a large number of nuclei, as though in an effort to put up a more effective conjoint resistance. Others regard them as originating from single cells. They may not be formed until necrosis begins in the tubercle. They may often contain tubercle bacilli. Though they are typically formed in tuberculosis, they are by no means peculiar to this disease. They are not seen in the more acute type of the disease where resistance is poor and in very mild infection which does not proceed to necrosis. Simultaneously with the above changes, there is also a leucocytic infiltration taking place in the periphery of the tubercle first with neutrophil leucocytes and soon after with the lymphocytic type.

As the process continues, degeneration of the tubercle soon sets in. It commences with necrosis of the central portion followed by caseation and softening of the caseated mass. These changes, necrosis and caseation, are believed to be caused by the toxic bacillary products (endotoxin), the degenerative process being aided also by the avascularity of the tubercle from thrombosis of the blood vessels. Caseation may not occur always; where resistance is high or virulence of the organism low it is absent. Caseation is associated with a very rapid multiplication of the bacilli, accounting for the presence of large numbers of them in the freshly liquefied material. The tubercles may also coelesce and form large masses. Another event that may occur is the erosion of a blood vessel, causing massive infection of the blood stream; it leads to rapid generalisation of infection, setting up acute miliary tuberculosis. Instead of softening and liquefaction, what happens sometimes is calcification or deposition

of calcium salts within the tubercle. The caseating mass is surrounded by fibroblasts and is eventually completely encapsulated and walled off from the surrounding tissue. This happens when the resistance of the individual is high or the virulence of the organism low: even in these so-called healed or quiescent lesions bacilli may still survive as can be proved by animal inoculation.

Human Tuberculosis. Tuberculosis is an infectious disease with a universal distribution. Mountainous areas, like the Ghats, the Himalayas and the Andes, and many isolated and remote islands were at one time completely free from it. This freedom was not due to any inherent resistance on the part of the population of such areas to the infection, but the disease did not exist there before and there was no opportunity to contract it. When subsequent contact with civilization also brought with it infection, tuberculosis spread among these virgin populations in epidemic form, causing the death of a large proportion of the people in a short time. The Gurkhas of Nepal and the Negroes of Africa are examples of such populations that were affected by high death rates on first contact with the disease. In such a community the chronic type, prevalent among the civilized nations, is not met with. What is seen resembles the acute form occurring among infants of civilized people. In the present there is no native race that has not come in contact with civilization and got tuberculised. India with its numerous unpenetrated areas and highly centralised urban areas combines the conditions obtained among the primitive races and those among the western nations who were in long contact with tuberculosis.

Whether tuberculosis makes any racial discrimination is very doubtful. The comparative figures for the Negroes and Whites in America indicate that, whereas practically there is an equality of incidence of clinical tuberculosis between these racial groups, the mortality is higher among the Negroes. But it should be noted that the economic and social conditions among the Whites are decidedly better than among the Negroes and data drawn on the basis of dissimilar conditions will lead but to erroneous inference. That the living tissues of one race are inherently more susceptible to *Myco. tuberculosis* than those of another is now little more than speculation. Till comparative studies of the distribution and mortality of tuberculosis between

different races living under identical conditions and exposed to the same risk of infection are made, no conclusion can be drawn.

At the present time tuberculosis is so widely disseminated that its actual incidence is very difficult to determine. In some countries, however, the prevalence of the infection has been defined by employing the tuberculin test aided by X-ray examination and post-mortem results. Thus, it has been demonstrated that in the large cities of Europe and America 90 per cent. or more of children at the age of fifteen and 97 per cent. of adults are infected. The infection is much less in rural areas. Quite obviously, all of them do not suffer from the disease. Only in a small proportion of them the infection is active and even among these only a small proportion suffer from clinical tuberculosis. Neither the proportion of active disease nor the mortality rate has been accurately estimated in the recent times.

In the Western European countries and the United States of America there has been a steady and relatively rapid decrease of tuberculosis from the middle of the last century with a corresponding fall in the mortality rate. No one factor is solely responsible for it; it is the outcome of a combination of causes. The phenomenal rise in the prosperity of these countries during this period is the greatest single contributing factor; others, such as the rapid strides made in the practice of preventive medicine and the great advances made in the early detection, isolation and treatment of cases, have also helped to accelerate this downward trend. Perhaps, the development of endemic immunity, *i.e.* the general resistance of the population to the disease as the result of long contact, has also a part in it.

In the United States, for example, the death rate from all forms of tuberculosis fell from 180·5 per 100,000 in 1900 to 43·0 in 1942. This decline displays certain peculiarities. The mortality rate is highest in the age group of one to two years, falls rapidly from this to rise again to a peak in early adult life, twenty to twenty-four years of age, and declines again with a small secondary peak between forty-five and fifty-five years of age; this last is referred to as the late adult tuberculosis. The recent decline in the mortality rate is most evident in the lowest age group and there is no doubt that it is due to the greater care paid on the infant's health. The highest mortality rate has thus shifted from the lowest age group to the young adult age group. The

distribution of mortality among sexes is also striking. Taking all ages into consideration, more men die of tuberculosis than women, but in the young adult age it is the opposite, more women dying than men. But the death rate of late adult tuberculosis is considerably more among men than among women. Accurate statistics regarding the incidence and mortality of tuberculosis are not available for this country.

The types of acid-fast bacilli that cause tuberculosis in man are the human and the bovine. That the avian type may rarely attack man is based on very slender evidence. The distribution of types in different countries varies somewhat. Taking all ages into account, the human type is responsible for the majority of infections in man. The bovine type accounts approximately for ten per cent. of human tuberculosis in Great Britain, more in Scotland than in England. In India tuberculosis due to the cattle type is considerably less due to various reasons. It is given as two per cent. Mixed infections with both types have been reported but are rare. Practically every organ of the body may be affected by tuberculosis. The main organs affected by the bovine type are the gastro-intestinal tract and the lymphatic glands, just as the lungs are particularly selected by the human type. Primary abdominal tuberculosis is mostly due to the bovine type, but secondary abdominal tuberculosis is usually due to the human variety. About 25 per cent. of meningeal and cerebral tuberculosis are in Great Britain due to the bovine type; the proportion is greater in children than in adults. The great majority of bone and joint tuberculosis and genito-urinary tuberculosis is due to the human type. About 98 per cent. of pulmonary tuberculosis are due to the human type and the remaining due to the bovine type. But recent investigations tend to show that, in Scotland at any rate, a greater proportion of pulmonary infection is bovine in origin. Lupus is almost equally represented by these two types, but in either the organism is of low virulence.

The human type of infection, especially the respiratory form of the disease, is transmitted through infected dust (Cornet), droplets and sprays (Flugge). The source of infection in the dust is the dried sputum, motion or urine containing the organism. Infected milk and milk products are the main vehicles for the bovine variety. Meat is seldom infected by the tubercle bacillus. Besides, it is never taken uncooked.

The portal of entry is chiefly the respiratory tract in the case of the human type of tubercle bacillus. Less commonly the alimentary tract, the genito-urinary tract and the skin form other routes of infection. Direct entry through the conjunctiva is very rare. Hereditary transmission does not occur; if infected by the tubercle bacillus the ova or spermatozoa are sure to be destroyed. Transplacental infection of the foetus, though a possibility, is exceedingly rare. Heredity may, however, play a part by transferring a diathesis to the offspring in the shape of lowered resistance and greater susceptibility to the infection. The bovine type enters the body almost exclusively through the alimentary tract. When tubercle bacilli are ingested, either in infected food or in infected droplets or dust, they gain entry into the faucial, pharyngeal and lingual lymphoid follicles and thence start by invading the upper cervical and retropharyngeal glands. Tonsillar tuberculosis is not uncommon but seldom clinically apparent. The mucous membrane of the stomach seldom permits penetration of the organism. Lower down, the lymphoid tissue of the intestines, *e.g.* the Peyer's patches, is first invaded, forming the primary focus from which further spread occurs.

In pulmonary tuberculosis two alternate routes of infection are possible: the respiratory route and the alimentary route. the majority of opinion now favours the respiratory route, infection taking place by the direct inhalation of infected dust, droplets or sprays of saliva or mucus. Part of the infection in the inspired air may be arrested in the nose and pharynx, wherefrom bacilli may penetrate through the mucous membrane into the local lymphoid tissue, establishing in it a primary focus of infection. The rest, consisting of the finer particles, reaches the lungs directly. This view is based on a number of factors: the type of the infecting bacillus, the anatomical distribution of the lesion in pulmonary tuberculosis and the results of animal experiments. According to the other view, infection occurs primarily through the mucous membrane of the alimentary tract. The infected material—dust, droplets or sprays—is ingested with food and the bacillus gets into the lymphatic system through the buccal, pharyngeal or intestinal mucosa, as mentioned above. In its course the organism may or may not cause detectable lesion at the site of entry. The tonsils, the cervical glands or the mesenteric glands may be primarily infected. Further spread from

these situations to the lungs takes place finally through the bronchial glands or *via* the blood stream. According to this view, therefore, infection of the lymphatic system is the primary event and the invasion of the lungs is secondary from the caseating glands. While there is no doubt that the path of infection for the bovine type is the alimentary tract, it is extremely doubtful whether the human type invades the respiratory system through this route. But the alimentary mucosa cannot be completely exonerated in all infections with the human strains and the latter may enter the body through this route and cause a proportion of the various non-respiratory forms of tuberculosis.

Another much debated question about the respiratory type of infection is the relationship between the childhood infection and clinical tuberculosis in the adult. One school holds that pulmonary tuberculosis in the adult is due to a recrudescence of latent childhood infection, while the other regards that adult tuberculosis is the result of a fresh exogenous infection rather than of a flaring up of an old childhood focus. The evidence, however, is in favour of the latter view. Reinfection need not necessarily be followed by immediate clinical evidence; even several years may elapse before the disease develops after reinfection.

The incidence of tuberculosis in a community and the rate of mortality from it are profoundly influenced by the social and economic factors. The spread of infection also greatly depends upon certain environmental conditions such as close contact, overcrowding, badly ventilated dwellings, occupational exposure to dust, particularly silica dust, and the like. Other conditions in the host, aside from poverty that depresses the general vitality and favours infection, are certain constitutional diseases like diabetes and chronic nephritis, some infectious diseases like measles, whooping cough and influenza, and repeated child birth. It has been mentioned above that in the large cities of Europe well over 90 per cent. of the populations show evidence of having been infected with tuberculosis. In the vast majority of them the condition is either healed or latent, enabling them to lead a normal life. This high degree of resistance has been the result of better economic conditions. Poverty with its attendant evils is undoubtedly the greatest single factor in undermining resistance The decline of tuberculosis and its mortality seen in the Western

European countries and America can be traced primarily to the effects of better economic and social conditions. How a disturbance of these factors can adversely affect the incidence of tuberculosis, is apparent from the tuberculosis statistics of the two world wars. In the first one the incidence of tuberculosis increased particularly in the European countries directly affected by war and the same tendency was again seen in several countries during the recent world war.

Community life affords ample opportunities for close contact which favours easy transmission of the infection. The rate of new infection in tuberculous families is undoubtedly higher than that in the general population. Such families with open cases constitute small endemic centres. They serve to transmit the infection through generations. This appears to be by far the commonest method of spread of tuberculosis in a community and it emphasises the importance of close contact. Whether this is only familial or is also influenced by genetic factors, is difficult to establish. So far as evidence goes, tuberculosis is not hereditarily transmitted and prenatal infection is a rarity.

Immunity. The nature of immunity in tuberculosis is not fully understood. There is no doubt that the tissues develop antibodies. The presence of agglutinins, precipitins, opsonins and complement-fixing antibodies have all been demonstrated in the blood of patients. They are present only in low titres. Inoculation with dead bacilli, and even tuberculin, generates such specific antibodies in the blood of experimental animals; but such antisera are devoid of any preventive or curative properties. What part they play in the mechanism of resistance to tuberculosis, is still obscure. Apparently they have none; no relationship between these antibodies present in the blood and the individual's resistance to tuberculosis has been as yet established. Nevertheless, it is fairly clear that a certain amount of active immunity, resembling that observed in syphilis, is undoubtedly developed in tuberculosis. A peculiar feature of this immunity is that it lasts only so long as there is a focus of infection in the body. The focus may be latent or active, but in any case the infected individual is immune to reinfection. This is an instance of *infection immunity*. The resistance to reinfection is manifested in the form of a hypersensitivity of the tissues. These are in an allergic state.

Certain observations lend support to the view that some degree of active immunity is developed in tuberculosis. In endemic areas tuberculosis in children usually runs a more acute course than in adults. In the non-infected infants under two years of age tuberculin reaction is usually negative, whereas in older children and adults it is positive in virtue of latent infection. When tuberculosis is newly introduced into a community, the type of disease occurring in the adult is the same acute form as in children, showing that the former have had no opportunity to develop any immunity as in the case of the adult population of endemic areas.

Koch's experiments on guinea-pigs form the basis of our knowledge regarding the mechanism of immunity in tuberculosis. He demonstrated that a tuberculous guinea-pig reacted to a fresh infection quite differently from a healthy guinea-pig. The subcutaneous injection of a normal guinea-pig with a small sublethal dose of tubercle bacilli produces a slight local lesion which heals in a few days. Ten to fourteen days later, a hard nodule develops at the site, which breaks down and continues as a discharging sore till the animal dies of progressive tuberculosis. The regional lymphatic glands show enlargement and caseation. But if a similar injection is given to a tuberculous animal, one that has been given a primary injection four to six weeks previously, instead of the late nodule formation a dark ecchymotic area is developed on the same day or the next, which sloughs out with explosive reaction and the shallow ulcer left heals promptly. The focal glands are not affected. This reaction is known as *Koch's phenomenon*. Animals may also be sensitised by the inoculation of attenuated or dead bacilli. The reaction can be elicited even when dead bacilli are used for the second injection. The markedly explosive reaction shows the hypersensitivity of the tissues to reinfection, while the quick healing and apparent localisation indicate the resistance. Localisation of infection, it may be rememberd, has been accepted as an expression of resistance. Koch's phenomenon, therefore, seems to support the theory that allergy is an expression of partial immunity. But this view is not accepted by all. The opponents maintain that allergy is a separate entity and that it has the reverse effect of immunity, affecting the allergic individual adversely (p. 316).

Vaccines made from dead cultures have no immunising value. Active immunisation with living attenuated organisms, like the

B.C.G., however, seems to confer a certain amount of protection. It is of short duration.

Tuberculin. The disintegrated protoplasm of the bacillus together with the products formed during growth in the culture medium constitutes tuberculin. Koch was the first to prepare tuberculin. He grew the tubercle bacilli for six to eight weeks in 5 per cent. glycerol broth, sterilised and evaporated the culture on water bath at 100° C. to one-tenth of its volume and had the contents filtered to remove the unlysed remains of the bacillary bodies. This clear, brown, syrupy filtrate he called *tuberculin*. It is now known as *original* or *old tuberculin* (O.T). This extract contains the endotoxin. One gram of tuberculin is contained in 1 c.c. of the extract. It is a stable product and, in the undiluted state, will withstand autoclaving for half an hour. When diluted it loses its potency in eight to ten days. It is standardised on the basis of its action on sensitised guinea-pigs. Subsequent to Koch's work, several methods have been employed in its preparation. These preparations produce similar, but less potent, effects to those caused by Koch's old tuberculin. Little or no difference has been detected in the tuberculins prepared from the human and bovine types, but tuberculin prepared from the avian type differs from the former. Koch's *new tuberculin* is made by grinding up the bacilli, obtained from the growth on solid media, in 50 per cent. aqueous glycerol until a uniform emulsion is obtained. It corresponds to a bacterial vaccine.

Tuberculin prepared from the growth on synthetic media has the advantage that it is free from extraneous protein constituents which are mostly responsible for non-specific reactions. The specific protein, or tuberculo-protein, is toxic and the skin reaction seems to depend mainly on it. It is now prepared in a purified form by fractionating the bacillus grown on synthetic media (Purified Protein Derivatives—P.P.D). Comparative studies of O.T and P.P.D would seem to show that the latter is quite as satisfactory as the former. The dose of P.P.D is 0·00005–0·005 mg.

The tuberculin test may be performed in several ways. Till recently Koch's old tuberculin was the one used in all these. It is rubbed on the scarified skin in Von Pirquet's cutaneous method; Moro's percutaneous method is to incorporate O.T in an ointment which is then rubbed on the intact clean skin; in Mantoux's

intracutaneous method it is injected intradermally; Calmette's ophthalmic reaction is done by dropping the tuberculin into the conjunctiva and Koch's old method is by injecting it subcutaneously.

Both the Von Pirquet's dermal test and the intracutaneous test of Mantoux are in common use. The latter is more delicate. The dose employed in these are too minute to affect tuberculous lesions. Calmette's ophthalmic test, on the other hand, causes serious reactions; so also Koch's original subcutaneous method. Therefore, these methods have been largely discarded. In the Von Pirquet's method, a drop of undiluted old tuberculin is placed on the cleaned skin of the forearm and at a distance one drop of 50 per cent. glycerine as a control. With a lancet a superficial scratch is made on the skin through each drop without causing bleeding, through the glycerine first and then through the tuberculin. In positive cases a bright red papule, at least 5 mm. in diameter, appears at the site within 24–48 hours, whereas the control area shows no change or only the traumatic effect. Later, the papule becomes dark red and disappears in a week. On pigmented skin the bright colour may not be very apparent.

The Mantoux test, also called the graded intradermal test, is usually done with one dilution;' a dose of 0·1 c.c. of a 1 : 1,000 dilution of old tuberculin is injected intradermally into the cleaned skin of the forearm. Even with this dilution troublesome reaction may be produced in those who are acutely sensitive to tuberculin. Hence some workers advocate an initial dilution of 1 : 10,000. The graded method is to test with several dilutions, 0·1 c.c. of 1 : 10,000, 1 : 1,000, 1 : 100 and 1 : 10 dilutions, one after the other at suitable intervals if the previous one was negative. A positive reaction is given by the appearance of an erythematous swelling, at least 5 mm. in diameter, at the site of inoculation within 48–96 hours. Non-specific reactions may appear but usually fade off within 48 hours.

Tuberculin reaction depends upon previous sensitisation with the bacillus. A healthy infant, which has never come in contact with the infection, can tolerate even a dose of 1 c.c. of neat old tuberculin. But in a tuberculous subject a dose of 0·01 c.c. produces severe reactions. Such reactions are local, focal and general and cause serious set back to the disease. The precise mechanism of these reactions is not fully understood.

It is allergic in nature and is probably an expression of immunity. Some substance, in all likelihood the tuberculo-protein, derived from the tubercle bacillus during the primary infection, produces hypersensitiveness of the tissues of the host. The tuberculin injected interacts with the sensitised tissue cells and gives rise to the reaction. Whether any special kind of toxin is formed as the result of this interaction between the tuberculin and the sensitive cells, is not known.

Tuberculin has been used for diagnostic and rarely for therapeutic purposes. In tuberculosis survey it is of great value. It is extensively used in the detection of tuberculosis in cattle. The best way of doing the tuberculin test in cattle is by the double intradermal method. Calmette's ophthalmic method is also employed.

A positive cutaneous reaction indicates the presence of a focus in the body. It may be very minute or even entirely healed. Hence, a positive reaction does not help to diagnose active from quiescent foci. A large majority of people above two years of age harbour latent infection, reacting positively. Therefore, except in infants under two years of age, the test has only a limited practical value in the diagnosis of clinical tuberculosis. In advanced cases, where the patient's tissues are unable to react, a positive reaction does not develop. During the course of, and convalescence from, certain acute infectious diseases, such as measles and diphtheria, tuberculin sensitisation tends to disappear. Extreme malnutrition, pregnancy and parturition also tend to reduce the tuberculin sensitiveness. In the very early stage of tuberculosis this allergic sensitiveness may be absent in some cases. Except in the above conditions, a negative reaction, especially with low dilutions by the Mantoux's technique, may be taken as sufficient evidence of non-tuberculisation.

Diagnosis. The microscopic examination for the tubercle bacillus is relatively simple. Large number of bacilli are usually found in acute spreading lesions, whereas they are scanty in chronic cases. The detection of acid-fast organisms in a suitably stained smear of the pathological material is generally sufficient for a positive diagnosis. The Ziehl-Neelsen is a satisfactory method. The search for the organism should be long enough and repeated before a verdict is given. It should be remembered that acid-fast saprophytes, which are widely distributed in nature, may be present as contaminants. Rarely other acid-fast orga-

nisms, like the leprosy bacillus and certain species of *Actinomyces*, are likely to cause confusion. The likely presence of the smegma bacillus in materials from the genito-urinary tract should be borne in mind. One negative result of microscopic examination by no means excludes tuberculosis in a suspected case and further investigations are necessary. Less than 100,000 bacilli per ml. of the material does not offer reasonable chance of detection by ordinary microscopy. Recently, claims have been put forward that fluorescent microscopy yields a higher percentage of success and is more rapid than the Ziehl-Neelsen technique; it is not yet adapted for routine use (p. 53).

The bacilli are not always uniformly distributed in the pathological product and, for this reason, the selection of a suitable portion from the specimen is important. This applies particularly to sputum. The purulent nummular portion must be selected for making smears. Prolonged examination may be necessary. If direct smears prove negative, any of the concentration methods, for example the antiformin method, should be employed before making smears. Specimen of urine, pleural or peritoneal fluid should be centrifuged and films made from the deposit. Preferably a twenty-four-hour specimen of urine should be examined. The tubercle bacillus is more resistant to the action of alcohol than the smegma bacillus, but the statement that the tubercle bacillus is alcohol fast whereas the smegma bacillus is not, is inaccurate, and the property of alcohol-fastness does net constitute a valid criterion for the differentiation of them (Bergey). If cerebrospinal fluid is allowed to stand for some time, a fine fibrinous coagulum is often formed; this should be removed, spread on a slide and examined after staining. Otherwise, the fluid should be treated like urine. The demonstration of the bacilli in pus is not so difficult as is sometimes said; prolonged examination often proves successful. The faeces should be treated with antiformin. It may be positive in both respiratory and non-respiratory tuberculosis. In the respiratory tuberculosis of children examination of throat swabs or stomach contents is frequently resorted to. The same may be done in adults with little or no sputum. The detection of tubercle bacilli in blood is extremely difficult. Sections from infected tissues are stained by the Ziehl-Neelsen method and examined (p. 72). Infected milk is examined by the direct method (p. 184), cultivation or by animal inoculation.

There are several methods of concentrating the bacilli from materials when they are scanty or difficult to detect due to their uneven distribution. The antiformin method is satisfactory. It also helps to kill the extraneous organisms and is particularly valuable in the isolation of tubercle bacilli from contaminated materials by culture or animal inoculation. Antiformin is a mixture of equal parts of liquor sodae chlorinatae (B.P.) and 15 per cent. sodium hydroxide. The cells and associated organisms are dissolved, while the vitality of the tubercle bacilli is left intact.

The contaminated material is mixed with three or four times its bulk of 15 per cent. aqueous antiformin, if necessary in a pestle and mortar, and digested in the incubator at 37° C. for fifteen to thirty minutes till it is thoroughly dissolved. If the material is very thick and tenacious, the proportion of the diluted antiformin has to be increased to ensure complete solution. The mixture is then centrifuged and the supernatant fluid is decanted. The sediment is then washed once or twice with sterile water and recentrifuged every time. Smears are made from the deposit and examined by the Ziehl-Neelsen method. For cultural or animal inoculation purposes the whole procedure should be done under aseptic conditions.

On account of the difficulties of cultivation, cultural procedure has not been followed as a routine measure for diagnosis. But now more attention is being paid to the cultural method of diagnosis. The suspected material, if uncontaminated, is directly inoculated on egg medium. If other organisms are present, the material is first treated by the antiformin method before seeding. Treatment with 4 per cent. sodium hydroxide (Petroff) or 6 per cent. sulphuric acid (Corper) for fifteen to thirty minutes or with 5 per cent. oxalic acid for thirty to sixty minutes also serves to destroy the contaminating organisms. The material is mixed with three or four times its volume of any of these and left in the incubator at 37° C. for the appropriate time; it is then centrifuged and the sediment is rendered neutral to litmus before inoculation. Growth of the tubercle bacillus sufficient to be seen by the naked eye may be expected at the end of a week; often it takes much longer. Cultural methods are often successful where direct examination has failed. The presence of 10-100 bacilli per ml. is said to give a successful culture. But there is no surety in any

case that the acid-fast organism seen or grown is the virulent variety.

Animal Inoculation. This diagnostic procedure takes a longer time than the cultural method, but it is the most delicate test. The guinea-pig is the animal of choice. This method also provides the best means of obtaining a pure culture of the organism. The suspected material is inoculated into the guinea-pig directly if it is uncontaminated. Otherwise, it is well to treat it before injection by any one of the methods mentioned above, in order to destroy the secondary organisms that may also be virulent to the animal and cause its death before tuberculosis has time to develop. Inoculations are made subcutaneously or intramuscularly into the thigh of the animal. The intramuscular route is preferable as the abscess which forms subsequently does not ulcerate through the skin giving rise to a discharging sore. Where possible, two animals should be inoculated. Following the local lesion, the inguinal glands are involved and then the iliac glands and thus the infection ascends and spreads through the lymphatic channels to other parts of the body. Finally, the animal dies in six to fifteen weeks. But it is advisable to kill one of the animals after three or four weeks. The survival period of the animal may, however, be very much longer and if this is not borne in mind a correct diagnosis may be missed in such cases by the premature killing of the animal. From the animal, material for culture is taken with aseptic precautions from a caseating lesion. Several tubes should be inoculated. It is a common experience that even when the number of organisms present in the material on microscopy is fair the number of colonies developed is few. Cultures on ordinary media are also put up simultaneously to exclude *Salmonella*, *Brucella*, *Pasteurella* and pyogenic infections which simulate tuberculosis. A direct examination of smears made from caseating material usually shows the bacilli and establishes the diagnosis. In doubtful cases portions of glands and spleen should be ground up and inoculated into a fresh animal. The examination of stained sections of glands and spleen also may help. The tuberculin test has been advocated for the detection of infection in the inoculated animal; but post-mortem examination remains the conclusive proof.

Allergic Test. The Von Pirquet and the Mantoux tests are the ones widely employed. The latter is more sensitive than the

former. The method of conducting these tests and the value of positive and negative reactions have been discussed in a previous section.

The Complement-Fixation Test. This is not of much practical value. Extracts made from the tubercle bacilli constitute the antigen and the general technique for doing the test is the same as that for the Wassermann reaction. Chronic and active cases of pulmonary tuberculosis generally give positive reaction, but in these cases easier methods of diagnosis are available. In early and in rapidly progressive cases the test is negative and it is in such cases that no reliable methods of diagnosis are available.

Prophylaxis. The prevention of tuberculosis, especially in this country, is a problem of immense magnitude and importance and but the barest outline of it can be given here. It can be dealt with under general and specific measures. Improvement of the economic and social conditions of the vast populace that live in perpetual semistarvation and extreme insanitation is the most urgent step. While it is a vast and complicated problem, it is the only effective way to strike at the root of the evil. Other measures to combat the spread of tuberculosis are: education of the masses about the infectious nature of the disease and how it can be avoided, improvement of general sanitation and enforcement of general hygienic laws, establishment of a safe milk supply, early detection, segregation and care of the tuberculous individual, regular examination of the contacts, and protection of the children of tuberculous families. The question of better housing with adequate ventilation and avoidance of overcrowding is contained in the problem of economic betterment.

Active immunisation with killed tubercle bacilli does not produce any immunising effect. Bacillary products, like tuberculins, also fail to confer any appreciable protection. But inoculation with live attenuated organisms seems to create a certain amount of resistance to infection both in the bovines and in the human beings. This is short-lived and of a low degree.

The B.C.G (Bacille-Calmette-Guerin) is a live attenuated vaccine introduced by Calmette and his co-workers. It is a bovine strain rendered avirulent by repeated subcultures (230 subcultures spread over a period of 13 years) on a bile-glycerine-potato medium, extending through several years. Extensive experiments with susceptible animals, including cattle,

guinea-pigs and monkeys and employing large doses both by the ingestion as well as the inoculation methods, have established its permanent avirulence. Though avirulent, it has not lost its antigenic power. The question of reversion in the tissues into virulent forms has not been proved so far. Calmette advocates immunisation of babies of tuberculous parents by the oral route (3 doses of 10 mg. each on alternate days) during the first ten days of life when the alimentary mucosa is easily permeable to the passage of the organism. Vaccination immediately after birth has the advantage that it forestalls natural infection. During the period of vaccination they must be removed to a place free from all sources of spontaneous infection. Such vaccinated populations have been reported as showing lower incidence and mortality rates. How far this lowering is traceable to active artificial immunity, requires further elucidation. In interpreting any experimental data on the subject, the possibility of a natural decline of tuberculosis, as occurring in the western countries, should be kept in view. Later evidence seems to show that vaccination by the intradermal or subcutaneous method is more effective than by the oral route. The former is preferable to the latter as the complications that may be produced are fewer and less serious.

Spahlinger's vaccine is another preparation credited with immunising virtue. Tubercle bacilli are grown in a medium consisting of tissue fluids derived from sources which form the natural habitat of the bacilli. They are then allowed to die out naturally in the medium by keeping the culture for a year or more. This constitutes the vaccine. Limited trials with this vaccine appear to have given encouraging results.

Some attempt has been made in the past to immunise with other types of acid-fast organisms. One such is the recent trial with the vole bacillus; it is still in the experimental stage.

The curative value of tuberculin is very limited. Very rarely it is used in the treatment of torpid cases with a view to activate the lesion. Extremely minute doses are employed to start with. The initial dose recommended is 0·1 c.c. of a 1 : 100,000 old tuberculin. This may be increased every two days by 0·1 c.c. After several months the patient is able to withstand a dose of 0·5 c.c. of the undiluted tuberculin and the course is finished by repeating the dose three or four times. It is very important to

remember that the dosage and the spacing are entirely governed by the patient's reaction. The chief governing consideration must be never to produce a febrile reaction. Chemotherapy has not made much headway in the treatment of tuberculosis. Gold salts are still under trial; so also the antibiotic streptomycin.

Tuberculosis in Animals. Animals in their natural surroundings seldom suffer from tuberculosis, but under conditions of captivity they may contract the infection. Domestic animals are often subject to spontaneous infection. Of these, the most frequent are cattle, pigs and fowls. Infection in pigs is commonly due to the bovine and avian strains acquired from infected cattle and poultry; they are also susceptible to the human type. Tuberculosis in fowls is invariably due to the avian type. With the exception of parrots and certain birds of prey, birds are very resistant to the mammalian types of tubercle bacilli.

Tuberculosis in cattle is almost exclusively due to the bovine variety of *Myco. tuberculosis*. Infection with the bird type may occur rarely. Cattle can with difficulty be infected experimentally with the human type, but infection so induced never leads to a progressive type of disease. They get the natural infection from other infected cattle or infected man. The incidence of bovine tuberculosis varies widely in different countries. In this country it is low: it is high, 40 per cent. or more, in Great Britain. In the latter country about 0·5 per cent. of all milch cows excrete tubercle bacilli in their milk. The disease is more frequent among the older animals and usually takes a chronic, slowly progressive course. The main brunt of attack falls on the lymphatic system. The most frequently affected organs are the lungs, pleura and the thoracic lymph glands. Tuberculosis of the udder is not infrequent, but it is important to remember that bacilli may be excreted in the milk even in the absence of any detectable lesions of the mammary glands.

Mycobacterium Avium. This organism was isolated from tuberculous lesions in fowls. In several respects it resembles the mammalian type. Branched forms are more frequent in the avian type than in the mammalian types. The avian type grows more freely and luxuriantly than the mammalian types and the growth tends to be moist or even slimy. The avian bacillus can be made to grow on simple nutrient agar or broth without any addition of glycerine. The addition of glycerol, no doubt,

improves growth; individual colonies are large, raised, hemispherical, creamy white, with a paint-like surface. On Dorset's egg medium the growth is creamy, confluent, slightly raised, with smooth regular surface. It does not form pellicle on fluid medium but forms a moderate viscous to granular deposit. The avian strains may develop a pink colour when grown on glycerol egg medium; on coagulated ox serum also they give rise to yellow or golden yellow growth.

The optimum temperature for growth is 40° C. with a range of 30°–44° C. *Myco. avium* is antigenically distinct from the mammalian types.

Myco. avium causes tuberculosis in domestic fowls and other birds, which are resistant to the mammalian varieties. Less frequently it attacks swine. Avian tuberculosis has been reported in other domestic animals including cattle; its occurrence in man is very doubtful. Experimentally it is pathogenic to birds and to a less extent to rabbits; guinea-pigs are resistant.

Mycobacterium Paratuberculosis. This organism, commonly called Johne's bacillus, causes a chronic slowly progressive enteritis in cattle, usually terminating fatally. A similar disease of sheep has also probably the same aetiology; the organisms in both are alike. Johne's bacillus is shorter than the tubercle bacillus and the ordinary forms stain uniformly. It is more difficult to grow than the tubercle bacilli and for primary growth, at least, requires the presence of other dead acid-fast bacilli or their extracts in the media; a glycerol egg medium with these is satisfactory. In cultures it resembles the tubercle bacillus.

The disease can be reproduced in calves, but the ordinary laboratory animals are refractory. The lesions in the intestinal wall are proliferative in type and show no caseation. As in leprosy, the bacilli are present in them in large numbers, usually packed inside cells. They are also present in the faeces. "Johnin reaction," brought out by injecting a culture filtrate, is an allergic reaction analogous to the tuberculin reaction. It is used in diagnosis.

Mycobacterium Muris. This is another mammalian tubercle bacillus recently discovered in the field vole, in which it causes a chronic infection resembling tuberculosis. In morphology it is more slender and often longer than the human bacilli; it is also reported to be pleomorphic. In cultural characters the

murine type resembles the tubercle bacillus, but it fails to produce pigment and to utilise glycerine for its growth. Both guinea-pigs and rabbits are susceptible to it, the latter more than the former. Preliminary work shows that immunisation of guinea-pigs and calves with small doses of live cultures of vole bacilli confers a certain amount of protection against the human and bovine types.

Tuberculosis in Cold-Blooded Animals. Acid-fast organisms with some resemblance to the mammalian types have been found to be the causative agents of certain natural tuberculosis-like lesions in cold-blooded animals. Such lesions have been found in frogs (*Myco. ranae*), turtles (*Myco. chelonei*), snakes (*Myco. thamnopheos*), fishes (*Myco. piscium*), and others. In cultures the cold-blooded types resemble closely the avian type. Their optimum temperature is 25° C. They are not pathogenic to warm-blooded animals; unfavourable temperature conditions are probably the preventive factors. Attempts to produce immunity with these organisms against the mammalian types in man and cattle have not been successful.

Acid-Fast Saprophytic Bacilli. They are found extensively distributed in nature and their importance lies in the fact that they may cause confusion during the identification of the pathogenic strains. They are found in milk, butter, manure, grass and other situations. They grow freely on ordinary media even at room temperature. The growth on solid media is dry, wrinkled, luxuriant and in most cases coloured yellow, pink or brick red.

The smegma bacillus is a commensal acid-fast organism found in the smegma of both sexes and on the skin. It is shorter and stouter than the tubercle bacillus. It is easily decolourised by alcohol. Its occurrence in the urine has to be remembered when searching for the tubercle bacillus in suspected cases of genito-urinary tuberculosis.

In staining reactions, growth requirements and cultural characters the saprophytes show certain differences from the pathogenic ones. But to rely on these solely for the differential diagnosis is not always safe. Animal inoculation offers the surest method. While the pathogenic strains cause progressive lesions in the guinea-pig, the non-pathogenic ones at most develop only localised lesions even after the administration of large doses.

Mycobacterium Leprae. *Mycobacterium leprae* was discovered in the leprotic tissues by Hansen in 1874. It is an obligate parasite of man. It is not found anywhere except in the human lesions and discharges. At certain stages of the disease the organism occurs in enormous numbers in the infected tissues, both inside and outside the tissue cells. The nasal secretions, rarely sputum, blood, urine and saliva may show the organisms.

Morphology and Staining. Leprosy bacilli are 1–8×0·3–0·5 micron in size and straight or curved with pointed or rounded ends. Generally, they occur in sheaves or clumps with the bacilli arranged in a parallel fashion often referred to as palisade or bundle of cigars arrangement. In staining reactions the leprosy bacillus resembles the tubercle bacillus, but the former is less acid-fast and less alcohol-fast and stains more readily by the Gram's method, retaining the violet stain. As a practical point, the relatively less alcohol-fastness of the leprosy bacillus should be remembered, particularly when staining sections. Leprosy bacilli also take the stain more uniformly and the beading is less frequent. When present, the granules are coarser than those of the tubercle bacillus. The tissue forms are non-capsulated, non-motile and non-sporing. *Myco. leprae* too has a lipoid envelope like the tubercle bacillus.

Cultivation. Although *Myco. leprae* is one of the earliest pathogenic bacteria to be discovered, we know nothing as yet about its growth requirements and other properties. Persistent efforts made all over the world have yielded very little concerning its physiology. No method suitable for its cultivation has been yet evolved and no susceptible animal discovered. Several claims of successful cultivation of the organism have been put forward. But it does not appear certain that the organism has ever been really grown in artificial media. The staining properties of the organism are retained for very long periods, even for three or four years or more, when a piece of infected skin is incubated in any medium like Hartley's broth. This property has also been observed if infected pus or sterile pus inoculated with the organism is incubated at 37° C. This may have led to the several reports of positive cultures.

As noted above, the leprosy bacillus, removed from the lesions, retains its staining properties for a very long time. Another peculiarity that has been noticed is that the bacilli show

with efflux of time an apparent elongation, thickening and greater granularity with enlargement of the granules. From these it is tempting to suggest that the organism may remain alive outside the body for a long time.

The growth of weakly acid-fast or non-acid-fast diphtheroids and other similar organisms in the culture tubes inoculated with leprous material has been obtained by many workers. The suggestion, therefore, has been made that the acid-fast bacilli found in the human lesions represent only one stage in the life-cycle of the organism and other stages appear outside the body. The existence of a non-acid-fast phase even in the tissues, accounting for the apparent absence of acid-fast bacilli in certain types of lesions, has been suggested.

Toxin Production. Nothing is known about the toxin production. In analogy with the tubercle bacillus, endotoxin may be the toxic factor causing the disease. It would appear that the endotoxin of the leprosy bacillus is very much less poisonous to the tissues than that of the tubercle bacillus. The toxic manifestations in a patient harbouring masses of leprosy bacilli in his body are relatively less severe than those produced by a far fewer number of tubercle bacilli. It is not uncommon to see patients suffering from lepromatous leprosy containing large masses of organisms in their lesions walking about with apparently no symptoms. Another explanation may be that the toxins are shut up locally and do not permeate into the vital tissues.

Pathogenicity. *Myco. leprae* is accepted to be the cause of leprosy in man. It is not pathogenic to animals. Rat leprosy, a natural disease of rats, is different from human leprosy. Successful infection of monkeys with infected material from leprotics has been recorded. The lesion developed in these animals is said to be a localised granuloma which regressed spontaneously.

Leprosy is an ancient disease prevalent in all the then known countries. It has been practically eradicated from Europe in the modern times. Now it is widely distributed in the tropics and subtropics. China, India, Africa, S. America and many of the Pacific islands are heavily infected. It is not entirely absent from the cold countries. Few cases occur in Iceland, Scandinavia and many other countries of Europe and in practically all the states of the U.S.A.

Infection seems to take place mostly in the early age. Race, as it is, does not make any difference in its incidence. Males are more often affected than females. The source of infection is the sick person discharging the bacilli from the breaking down nodules, in the nasal secretions and probably in the saliva and urine. Very little is known about the exact mode of transmission. How long the leprosy bacillus can remain viable outside the host tissues, is not known. For this reason, whether the disease can be transmitted indirectly through the agency of infected food, fomites and other articles, also remains problematic. Leprosy, like tuberculosis, is not a hereditary disease. The spread of infection is believed to be through contact. Prolonged and intimate contact is said to be necessary. As in tuberculosis, a familial transmission is definitely traceable in leprosy. Regarding the actual route of entry, again, there is no definite knowledge. Probably, the nasal and the pharyngeal mucosa and the skin serve as portals of entry. The lobule of the ear is believed to be a common site of entry. Apparently, there is no observable primary lesion, unless the ulceration of the nasal mucosa found in a fair percentage of cases is considered as such.

The incubation period is long, varying from a few weeks to several years. There is some evidence to show that, before clinical symptoms appear, there is a general dissemination of the bacilli in the skin. The organism has been found in the internal viscera without actual lesions. Although most of the organs or tissues in man may be affected, the skin and the peripheral nerves are the ones chiefly involved. As in tuberculosis, the essential lesion is a granuloma, the so-called leproma. Lepromata may vary in size from minute to big nodules. Histologically, large numbers of fibroblasts, lymphocytes and large vacuolated, mononuclear cells, often crammed with acid-fast bacilli, are seen. The last type of cells are known as *lepra cells.* They are reticulo-endothelial in origin. They are pale foamy in appearance due to the presence of a high lipoid content. Giant cells may be encountered, but they are different from the giant cells of tuberculosis. As against the tubercle, the leproma is provided with blood and lymph supply. Hence caseation does not occur. There is infiltration of the affected nerve bundles with granulomatous tissue followed by fibrosis and eventual pressure atrophy of the nerves. When the peripheral nerves form the

seat of lesion, relatively few organisms are present and it is difficult to show them. Consequently, the neural or the anaesthetic type is very much less infectious than the cutaneous or the nodular type. The degenerative changes in the implicated nerves lead to trophic disturbances and atrophy of bones, particularly of the small bones of the hands and feet, and skin and trophic ulcerations are a common feature of the disease. The bacilli are usually absent in such ulcers.

Leprosy is a chronic disease lasting for several years. Considerable mutilation and disfigurement are left in burnt out cases. By itself it is seldom fatal; but it may give rise to complications or pave the way for intercurrent infections which end fatally. Acute exacerbations during the course of the disease are common and, if not adequately controlled, adversely affect the course. A temporary lowering of the general resistance of the body from any cause is probably responsible for their production. On the other hand, increase in the general resistance hastens improvement.

Diagnosis. The only reliable method is the finding of the organism in suitably stained smears or sections examined under the microscope. This is not always possible, especially in the anaesthetic type of the disease, when reliance has to be placed entirely on clinical symptoms. Cultural and animal inoculation methods are of no avail, except as negative evidence. Sometimes the question may arise whether an acid-fast organism observed is the leprosy bacillus or the tubercle bacillus. Morphologically the former is shorter, stouter, straighter and more solidly stained than the latter. The vast numerical superiority, the characteristic arrangement, the intracellular position in what are known as the lepra cells are other differentiating points. The leprosy bacillus is decolourised with acid-alcohol in a much shorter time than the tubercle bacillus. When there is a suspected involvement of the lungs in leprosy, tuberculosis can be ruled out by negative cultural and animal inoculation results. But both infections may coexist.

The smears for examination have to be taken carefully. The organism is not usually found in the epidermis or in the superficial layers of the mucous membrane. The usual procedure is to take a skin clip, not less than 2 mm. thick, with a pair of curved scissors. With the forceps used for holding the skin, the clip is

squeezed while making films on clean slides from the cut surface. The skin clip may, when considered necessary, be used for cutting sections. The site chosen for taking the clip is the surface of a nodule, the advancing edge of a lepromatous patch or the lobule of the ear. Even in the absence of thickening, smears from the last site may give positive results. If there is ulceration of the nasal septum, smears are made by means of swabs taken from the surface of the ulcer. In the absence of ulceration the anterior third of the septum is gently scraped with the point of a sharp knife till it bleeds slightly. The scrapings on the knife are directly smeared on clean slides. The demonstration of the bacillus from anaesthetic patches is regarded as almost impossible, but reports to the contrary have been published. The organisms are not usually present in the discharges from trophic lesions.

The complement-fixation test has been tried in the diagnosis of leprosy. But it is of no practical value. The erythrocytic sedimentation test has been extensively employed in the diagnosis but with no satisfactory results. It has been said that leprosy, especially certain types, gives a positive Wassermann reaction. This is extremely doubtful and the percentage of Wassermann positives among leprotics does not appear to be higher than that among the general population.

Leprotics may develop a hypersensitivity to the body substance of acid-fast bacilli, including the leprosy bacillus. It can be demonstrated by the *lepromin* test which causes an allergic reaction. Lepromin is a substance prepared from leprous nodules. In the present form, it is a group reaction and has little or no diagnostic significance.

Prophylaxis. No specific prophylactic measures are available against leprosy and the problem of prevention is at present largely based on early detection, isolation and efficient treatment of the sick persons. Much success has attended the isolation and segregation of patients in certain parts of the world, notably in Norway. Close contact is believed to favour spread; therefore, measures against it should be taken. Evidence suggests that young age is the most vulnerable period. Protection of children from sick parents and relations is, therefore, an important part of prevention.

Treatment. Different types of vaccines have been used in the treatment of leprosy. They have been prepared by grinding

up whole leprous nodules, from bacilli separated from lesions or from supposed cultures of *Myco. leprae* and even from other organisms like the tubercle bacillus and certain *Actinomyces*. The improvement with these, if any, is very limited.

Many drugs have been employed in the treatment of leprosy, but none, except choulmoogra oil and its derivatives, has stood the test of trial. Promin, diasone, streptomycin and others are on trial.

Mycobacterium Leprae Murium. The organism of rat leprosy was discovered by Stefansky in 1901 in the tissues of infected rats. In morphological features and staining properties it resembles the human leprosy bacillus. The organism is found in large numbers in the specific lesions both intracellularly and extracellularly. It has not been as yet successfully cultivated. The disease manifests itself in the rat in two forms—the glandular type and the musculo-cutaneous type. It has no relationship with human leprosy. Rats are immune to infection with leprous material from man. Rat leprosy has a world wide distribution and is found among *R. rattus* and *R. norvegicus*. Experimental transmission of the disease to healthy rats by the transplantation of infected material from the sick ones is possible. By the same method the infection has been recently transmitted to white rats, mice and guinea-pigs. Other animals are insusceptible.

CHAPTER XXIX

PFEIFFERELLA

The generic characters of *Pfeifferella* are: short, slender rods with rounded ends, sometimes forming threads and show and a tendency to branching: motile or non-motile: stain eal injection often irregularly and exhibiting a tendency to ad of infection to Gram-negative. The genus contains only ammation and swelling *mallei* and *Pfeifferella whitmori*: both wo or three days. This is animals and man. or *Straus reaction*. It is followed

Some authors use the out ten days.
more correct, generic nam wide infection, but of restricted occur- some doubt about its e disease is rare in this country. It is an restricted biochemical marily affecting the equines. How it spreads bacillary forms, sor not clear. The portal of entry is the respiratory akin to *Brucello* Two clinical types are seen, glanders with major tendency to brai the lungs and farcy or cutaneous glanders. The the *Mycobacta* y be acute, subacute or chronic, but each may run the view tha her. Latent infection has also been reported. The hand and *A* esion is a granuloma; small nodules are formed at the bacillus ir site and subsequently in the lungs and internal organs. general a entually ulcerate, giving rise to a discharge. Large num-

Pfeif bacilli are present in the lesions and discharges. In the (L., mall pe they are less numerous. The disease is invariably in 1882
site fou landers is uncommon in man. It occurs chiefly in persons other a ome in close contact with horses, asses and mules; hence

M narians, ostlers, grooms and coachmen are the usual victims. slender mission of infection is direct, through contamination of with sions or wounds with glanderous discharge from sick animals. and ind from man to man is extremely rare. Primary infection but gr also occur through the nasal or oral mucosa. Laboratory into l ers are very prone to get the infection: the glanders organism involu ry infective and, next to *Past. tularensis*, is the most dangerous bacill leal with.

is not stained very readily; carbol fuchsine and Loeffler's methylene blue give good results. Uneven staining and bipolar staining are common. In tissues granular appearance, stained areas alternating with unstained areas, is characteristic. Culture forms, especially when old, may also stain irregularly, with a tendency to bipolar staining. The organism is Gram-negative and non-acid-fast.

Growth Requirements. Oxygen is necessary for growth, no growth occurring when it is strictly excluded. No growth was obtained below 20° or above 44° C.; the optimum temperature is

rats. In mor

bles the human racters. *Pf. mallei* is a slow grower, although it large numbers in the ure media. The addition of glycerol (4 per extracellularly. It has not roves growth. A slight acid pH is The disease manifests itself in the vex, slimy, whitish colonies are type and the musculo-cutaneous ty 5–1 mm. diameter after two with human leprosy. Rats are immu ger, opaque, granular and material from man. Rat leprosy has a nies are also often noted. and is found among *R. rattus* and *R. nor* w, turning in four days transmission of the disease to healthy rats by olate-brown. Broth of infected material from the sick ones is possi bin surface growth method the infection has been recently transmitt nolysis on horse mice and guinea-pigs. Other animals are insusce

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Toxin Production. No diffusible toxin is elaborated by the glanders bacillus. Presumably the endotoxin is the disease-causing agent.

Pathogenicity. It causes glanders or farcy in horses, asses and mules; from these animals it may spread to man. Experimentally the disease can be readily reproduced in the equines and goats and less readily in sheep; pigs and cattle are resistant. Of the laboratory animals, guinea-pigs are very, and rabbits, rats and mice less, susceptible. Subcutaneous injection into guinea-pigs causes abscess formation locally, subsequent generalisation and death in about three or four weeks. The intraperitoneal injection into a male guinea-pig leads to the rapid spread of infection to the tunica vaginalis, causing acute inflammation and swelling of this structure and the testicles in two or three days. This is known as the *testicular reaction* or *Straus reaction*. It is followed by death of the animal in about ten days.

Glanders is a world wide infection, but of restricted occurrence anywhere. The disease is rare in this country. It is an infectious disease primarily affecting the equines. How it spreads among animals, is not clear. The portal of entry is the respiratory tract and skin. Two clinical types are seen, glanders with major involvement of the lungs and farcy or cutaneous glanders. The condition may be acute, subacute or chronic, but each may run into the other. Latent infection has also been reported. The essential lesion is a granuloma; small nodules are formed at the primary site and subsequently in the lungs and internal organs. They eventually ulcerate, giving rise to a discharge. Large number of bacilli are present in the lesions and discharges. In the skin type they are less numerous. The disease is invariably fatal.

Glanders is uncommon in man. It occurs chiefly in persons who come in close contact with horses, asses and mules; hence veterinarians, ostlers, grooms and coachmen are the usual victims. Transmission of infection is direct, through contamination of abrasions or wounds with glanderous discharge from sick animals. Spread from man to man is extremely rare. Primary infection may also occur through the nasal or oral mucosa. Laboratory workers are very prone to get the infection: the glanders organism is very infective and, next to *Past. tularensis*, is the most dangerous to deal with.

Infection through the skin is far more common in man. Circumscribed inflammatory nodules are formed at the site, whether in the skin or the nasal mucosa. In acute cases the infection becomes rapidly generalised, forming multiple pustular nodules on the skin and subcutaneous tissues and in the lungs or internal organs. Spread occurs *via* the lymphatics. Mucopurulent discharge from the nose, high fever and severe prostration are prominent symptoms and death may occur in seven to ten days. Chronic cases may go on for weeks or months and finally succumb to the infection. Recovery is exceedingly rare. Latent infection in man also has been reported.

Diagnosis. The microscopic examination of stained films, prepared from the pus or discharge taken from the patient, may reveal the presence of the characteristic bacilli. But it is not always satisfactory. Moreover, a negative finding does not exclude glanders. The isolation of the organism from the pus or discharge and the subsequent identification should, therefore, be proceeded with. A honey-coloured growth is a characteristic feature; the specific agglutination will clinch the diagnosis. Animal inoculation is another reliable method. If the original material or an isolated culture is injected intraperitoneally into a male guinea-pig, Straus reaction, the testicles becoming red and swollen, is obtained in two or three days. It is accompanied by severe general symptoms and the animal dies subsequently, showing characteristic lesions in the spleen, lungs, liver and other internal organs. The organism is found in the lesions, especially the inflamed tunica vaginalis, in considerable numbers. If the original material contains also extraneous organisms, it is rubbed into the scarified skin of the animal.

The *mallein test* is an allergic test analogous to the tuberculin and brucellin tests (p. 327); it is employed only in veterinary practice. *Mallein* is the sterilised and concentrated filtrate from a four weeks old glycerol broth culture. It is prepared in the same way as tuberculin. It is usually injected subcutaneously. A local reaction accompanied by a rise of temperature and often profound constitutional disturbances constitutes a positive test.

Serological tests are seldom applied in the diagnosis of glanders in man. The complement-fixation test is more reliable than the agglutination test. These are sometimes used in the diagnosis of animal glanders, particularly the former to show up

chronic and latent cases. In assessing the results of agglutination reaction it should be noted that the serum of normal horses agglutinates the glanders bacillus up to a titre of 1 : 300.

Prophylaxis and Treatment. The elimination of the disease from the equine animals is the surest method. In certain countries, like England, legislative measures have been taken with great success. Next is the avoidance of contracting the infection. Vaccination and serum treatment are both devoid of any value, either prophylactically or curatively. Chemotherapy likewise does not help.

Pfeifferella Whitmori. *Pf. whitmori* is the causative agent of melioidosis. It was discovered by Whitmore and Krishnaswami (1913) from a glanders-like disease in man in Rangoon. In certain respects it resembles the glanders bacillus but is motile. It grows readily on ordinary media. A brownish growth on potato is likewise obtained in this. Acid is produced in lactose, glucose, saccharose, dulcite and mannite; gas is not formed. Indole is not produced; gelatin is liquefied. Serologically *Pf. whitmori* closely resembles *Pf. mallei*; the former appears to constitute a homogeneous group. The organism is virulent to guinea-pigs and rabbits.

Melioidosis occurs in certain restricted areas in the Malay States, Indo China, Burma and Ceylon. It is epizootic among rodents and from them man gets the infection. How the infection spreads, is not known. The disease in rodents is similar to glanders, but in man it is usually a septicaemia or pyaemia. There may be localised suppurative lesions in the internal organs. The organism may be isolated from the blood, urine or pus. Serologically Whitmore's bacillus is related to *Pf. mallei* (p. 580). It also gives rise to Straus reaction in male guinea-pigs.

CHAPTER XXX

ACTINOMYCES

The actinomycetes probably form a link between the less differentiated *Eubacteriales* on the one hand and the more advanced fungi on the other. They are composed of cells usually elongated, frequently filamentous with a tendency to form branches, and in some genera forming a definite branched mycelium. The order *Actinomycetales* is divided into two families, the *Mycobacteriaceae* which include the mycobacteria and the corynebacteria, and *Actinomycetaceae*. The latter contains three genera, *Leptotrichia*, *Erysipelothrix* and *Actinomyces*. A new genus, *Proactinomyces*, including all the pathogenic species, has been created recently, but it has not received wide acceptance.

As stated above, the organisms belonging to the genus *Actinomyces* are higher forms of life than the ordinary bacteria. There is still some uncertainty about their systematic position; some regard them as transition forms between the bacteria and the fungi. Several names have been given to them from time to time—*Streptothrix*, *Nocardia*, *Discomyces* and others, but they are now largely given up. The genus was defined by the Committee of American Bacteriologists (1920) as follows: "organisms growing in the form of a much branched mycelium, which may break up into segments that function as conidia. Sometimes parasitic, with clubbed ends of radiating threads conspicuous in lesions in the animal body. Some species are microaerophilic or anaerobic: Non-motile". The branching may be lateral and dichotomous. Granules are often found inside the mycelial threads. With a few possible exceptions, all are Gram-positive. Some are acid-fast. These organisms are chiefly found in the soil as saprophytes. Many are parasitic on plants and animals and a few are pathogenic.

The mycelium in the parasitic species consists of an interlacing mass of branched filaments or hyphae which tend to radiate from a central knot towards the periphery where they may exhibit

club-shaped swellings. Hence the name "ray fungus" sometimes applied to it. This type of growth is well marked in lesions but not in culture. In fact, it may be absent in some of the aerobic pathogens. The filaments are straight or wavy, slender, measuring 0·5–0·8 micron thick and extremely pleomorphic, often breaking down into bacillary or coccoid forms. Sometimes they may even simulate streptococci in appearance. Two types of terminal swellings are encountered, the "tissue clubs" and the "culture clubs". The tissue clubs are very prominent features. They are all turned to the periphery of the mycelium. In older mycelia these clubs may constitute the sole surviving structure owing to the degeneration of the central portion of the colony and the formation of a structureless mass. They seem to be accretions from the tissues and are Gram-negative. They are weakly acid-fast. Another view is that "club" formation is the result of a swelling of the sheath of the filament at the extremity as a protective reaction on the part of the organism against the tissues of the host, a sort of defensive mechanism. The culture clubs are true swellings of the mycelial ends and do not show difference in staining. They are less conspicuous.

On the ground of oxygen requirement these organisms are classified into two groups: the aerobic and the anaerobic. The aerobic species, also called the Bostroem types, are found as saprophytes in the soil and elsewhere or as parasites on plants, grains, grass, etc. Members of this group are frequently found as secondary invaders in lesions of the upper respiratory tract of man, mouth, genitalia and skin. A few species of the aerobic type are acid-fast, but this property is weak and a one per cent. mineral acid should be used instead of the usual concentration. The production of pigment is characteristic of many species; almost any colour may be produced. Two species, *Act. madurae* and *Act. asteroides*, cause disease in man.

The anaerobic ones, or the Wolff and Israel types, are strict parasites and include *Act. bovis* which causes a granulomatous disease in cattle, sheep and occasionally in man. The anaerobes are all non-acid-fast.

Actinomyces Bovis. *Act. bovis* was discovered by Bollinger in 1877 and at his instance it was studied and described by Harz in the same year. The latter also gave the parasite its name *Actinomyces* or ray fungus on account of the ray-like fashion in

which it grew in the tissues. Wolff and Israel (1891) appear to have been the first to cultivate and isolate the organism in pure culture. *Act. bovis* is a strict parasite found in the lesions of actinomycosis in cattle and man.

Morphology. The appearance of the organism in animal tissues differs markedly from that in the culture media. In tissues growth occurs in the form of mycelial masses showing as characteristic pale yellow granules "sulphur granules" or *Drusen*, about 0·25–1 mm. in size. These granules are found in the pus discharged from the lesions. Each granule consists of a mass of tangled mycelium exhibiting a tendency to radiate to the periphery, where they end in characteristic club-shaped swellings. The filaments are very pleomorphic and branching. Associated with these are also seen large number of rod-shaped and coccoid bodies. Club formation is usually more common in the bovine than in the human lesions. In the culture also the organism is very pleomorphic; long branching elements of varying length, bacillary forms and coccoid forms are all seen. Club formation is not a marked feature under artificial conditions. The presence of intracellular granules is common in both tissue and culture forms. The organism is non-motile, non-acid-fast and Gram-positive. But the tissue clubs are Gram-negative.

Growth Requirements. The parasite is more microaerophilic than anaerobic. A lowered oxygen tension with 5–10 per cent. carbon dioxide pressure would appear to be the best for growth. The optimum temperature is 37° C. and the optimum pH 7·2–7·6. Not much is known about its nutritional needs. It grows fairly well in ordinary media. From this it would appear that the organism is not exacting in its nutritional requirements. Enrichment with glucose (one per cent.), blood or serum improves growth somewhat. But, even in the best of conditions *Act. bovis* is a slow grower.

Cultural Characters. Under suitable gas phase growth occurs on ordinary media, but poorly and sparsely. On agar small, greyish white, convex colonies with a smooth dull surface and entire edge appear, which grow up to a diameter of 1·5 millimetres in the course of about three weeks. The older colonies have an irregular nodular surface and lobate edge. They may grow into the medium and come out entire when tried to remove. Emulsification is not much difficult. Glucose agar shake culture

does not show any growth for about one centimetre below the surface. Below this are seen scattered about irregularly round, tiny, opaque, greyish white colonies. The organism does not grow on MacConkey's medium. In broth growth occurs as white granules which sediment, leaving a clear supernatant fluid. There is no surface growth.

Biochemical Reaction. Glucose, maltose, lactose, saccharose and salicin are fermented with the production of acid without gas. Litmus milk is not changed. No indole is produced nor hydrogen sulphide. Nitrates are reduced. Gelatin is not liquefied. No haemolysin is formed nor soluble pigment. The biochemical reactions have no practical value.

Resistance. Its resistance to adverse influences is only of the same order as that of other vegetative bacteria. Moist heat at 60° C. kills the organism in 10–15 minutes. Culture may remain alive for about one to three weeks. The preservation of strain may be done in glucose agar shake culture.

Pathogenicity. The organism causes actinomycosis in man, cattle and less frequently in swine. Only very mild lesions are produced by experimental inoculation into laboratory animals. Such lesions are benign and heal after varying periods.

Actinomycosis. Actinomycosis is a rare disease. It is much more prevalent among cattle than among man. How the infection is transmitted, is not known. Probably it enters through the mucosa of the mouth. The majority of cases in cattle are found in or about the head, the lower jaw and the tongue being particularly picked up by the parasite. The disease may also occur in other parts of the body, such as the pharynx, lungs, lymph glands, udder and liver. Clinically the lesion is a hard tumour which softens and disintegrates the adjoining tissues, including the bone. Simultaneously with destruction there is also formation of new tissues. The tumour extends gradually to the contiguous tissues. Metastasis is uncommon and generalisation rare; when it occurs, the blood stream is the more common route than the lymph. The disease is very chronic and death is due to mechanical causes than toxaemia. In macroscopic appearance the lesions in the lungs and udder often simulate tuberculosis. The essential lesion is a granuloma and a histological section shows epithelioid cells and often giant cells. The parasite is present in the tissues as mycelial masses or granules.

The discharge from the lesion is yellowish or blood stained and contains the characteristic small yellowish granules which can be readily shown to be colonies of the organism. A small Gram-negative, non-motile bacillus, *Actinobacillus actinomycetum comitans*, frequently occurs in association with the mycelia (p. 592). By appropriate methods pure culture of it can be obtained. Its actual role in the infection is not known. When inoculated alone into experimental animals, it fails to produce any lesion.

The mode of infection in man also is obscure. The disease, whether in cattle or in man, does not appear to be very infectious. Contact with cattle may favour infection. Probably the oral mucosa may be the portal of entry. Organisms closely resembling *Act. bovis* are sometimes found as commensals in the upper respiratory tract, mouth and intestine and it is possible that the infection is endogenous.

In man the disease is more confined to the soft parts and involvement of the bone is far less frequent than in the cattle. About two-thirds of cases are in the cervico-facial regions. The thoracic and the abdominal viscera are the next frequent sites. Though the disease usually spreads by contiguity, metastasis *via* the blood and lymph channels may also occur, followed by the setting up of secondary foci commonly in the brain, kidney, spleen and lungs. Abscesses are formed in these organs. Discharging sinuses form a common clnical feature. The characteristic granules, though present in the lesions, may be sometimes absent from the discharge. The disease may often be less chronic in man than in the cattle. Death may be due to embolism or secondary infection. Spontaneous cure may take place. There is no evidence of any effective immunity response in actinomycosis. The development of hypersensitivity to the parasite has been reported recently.

Diagnosis. The diagnosis of actinomycosis is not difficult and depends upon the demonstration of the parasite in the pus or sputum followed by its isolation and identification. The pus is spread in a sterile Petri dish, if necessary mixed with some sterile salt solution or water. The granules are easily seen. One or two granules are picked up and crushed on a slide with another slide, stained by the Gram's method and examined. The Gram-positive mycelium, presenting the characteristic appearance, is seen. Even in the absence of granules smears may show mycelial

threads. A few granules are then picked out by means of a sterile pipette, washed in sterile saline twice or thrice and finally inoculated into a number of solid and liquid media containing glucose, serum or blood They are then incubated anaerobically, after which isolation is proceeded with.

Actinomyces Asteroides. Originally isolated from a brain abscess (Eppinger), it has been subsequently reported from a number of pathological conditions. It is an aerobe. It is Gram-positive and weakly acid-fast. The mycelial threads are thicker; they readily break up, especially in fluid culture, into bacillary forms. The organism grows well on ordinary media. Older colonies may be pigmented yellow or pink. Lesions of the lungs, pleura and peritoneum due to this parasite have been several times reported. A chronic affection of the lungs with *Act. asteroides* closely counterfeits tuberculosis. In smears stained by the acid-fast method, only bacillary forms of the parasite are seen and are then scarcely distinguishable from tubercle bacilli. Culture and guinea-pig inoculation, however, provide ready means of differentiation. *Act. asteroides* is pathogenic to rabbits and guinea-pigs.

Actinomyces Madurae. This parasite is responsible for a chronic granulomatous disease, principally of the foot, called mycetoma or Madura foot. But other species of actinomyces and certain moulds may also cause mycetoma.

Morphology. The mycelial filaments are 1·0–1·5 microns in thickness. The ends break up into ovoid conidia, particularly marked in artificial culture. There is true branching. The tissue forms appear as interlacing network of mycelial filaments, often radially arranged and ending in club-like swellings. In culture it grows as long, non-segmented, branching filaments. A mycelial arrangement is not conspicuous. The organism is non-motile, Gram-positive and not acid-fast. The tissue forms may show irregular staining, but the culture forms take uniform staining.

Cultivation. *Act. madurae* is an aerobe. Its optimum temperature is 37° C. On agar small, round, convex colonies are formed. They are greyish yellow, opaque with a glistening surface, adherent to the medium and very difficult to emulsify. In broth growth is poor, showing clear supernatant fluid and white granular deposit. The addition of glucose or glycerol improves growth. A pigment is often formed, which is insoluble.

Resistance. The organism is not more resistant to adverse influences than the vegetative bacteria. Moist heat at 60° C. destroys the microbe in five minutes.

Pathogenicity. *Act. madurae* causes mycetoma in man. It is not known to be naturally pathogenic to animals. Inoculated subcutaneously into rabbits and guinea-pigs, the organism produces a small local nodule which regresses after a month.

Mycetoma or Madura foot is a disease of the tropical and subtropical regions, but cases have also been reported from certain countries in the temperate zone. Its greatest prevalence is in India and some countries adjoining the Mediterranean basin. Cases were first reported about the year 1842 from Madura, hence the name.

Besides *Act. madurae*, several other species of *Actinomyces* may also cause the disease. The more common of these are *Act. somaliensis*, *Act. asteroides* and *Act. bovis*. Madura disease may also be caused by certain moulds (*Hyphomycetes*) belonging to a number of genera, such as *Aspergillus*, *Penicillium*, several species of *Madurella*, and others. Of these, *Madurella mycetoma* is the commonest. It is usual to classify mycetoma into two aetiological groups: the actinomycotic type caused by *Actinomyces* and maduramycoses due to moulds.

The disease affects chiefly the foot; rarely the hand and other parts of the body may be affected. There is undoubtedly some relationship between the incidence of the disease and habitual locomotion barefooted. The organism probably enters through the skin and a history of injury or abrasion, for example the prick of a thorn, can invariably be obtained. A small subcutaneous swelling appears usually on the sole of the foot. It soon ruptures, giving rise to a discharging sore. The infection gradually extends, burrowing into the deeper tissues. Considerable swelling and distortion of the foot, suppuration and formation of nodules and multiple sinuses, discharging an oily often foul-smelling pus, are the characteristic features of the disease. The parasite is present in the lesions in large numbers.

The mycelial granules are found in the infected tissue and discharge. They may be up to 1 mm. in diameter, often larger than the granules of *Act. bovis*. The granules may be white, yellow or black in colour. The yellow and white types are usually associated with the actinomycotic type of mycetoma and the black

granules with maduramycoses. But it is unsound to classify the disease on the ground of colour production by the mycelium, since granules having the same colour may be produced by widely different species and conversely the same species may at times produce differently coloured granules.

Clinically no differentiation can be drawn between the actinomycotic Madura foot and that caused by moulds. Probably, in the former there is a greater tendency to bone involvement.

Diagnosis. The mycelial granules are present in the discharge and the parasite may be demonstrated as in the case of actinomycosis. However, the granules may be very hard and gritty and treatment with 20 per cent. caustic soda solution may be necessary to dissolve out the debris and pigment. It may be remembered that club formation may not be a conspicuous feature in mycetoma. The cultivation of the organism may be done as for actinomyces, but when maduramycosis is suspected Sabouraud's medium should be employed.

Treatment. The administration of heavy doses of potassium iodide has some value. Penicillin therapy has been claimed to yield good results in some cases. But when there is considerable tissue destruction, surgical removal of the affected part is the only effective measure.

Actinomyces Muris. This is an actinomyces originally isolated from cases of rat-bite fever. It is variously known as *Streptothrix muris ratti*, *Haverhillia multiformis*, *Actinomyces muris-ratti* and *Srteptobacillus moniliformis.* Some authorities regard it as responsible for one type of rat-bite fever in man—sometimes described as erythema multiforme or Haverhill fever. It is a normal parasite of rats and mice and is present in the nasopharynx. The organism is described as Gram-negative; it is very pleomorphic and shows bacillary, coccobacillary and filamentous forms, the last showing characteristic fusiform swellings. There is no doubt that it has been several times isolated from cases of rat-bite fever, but its real relationship with the disease awaits further elucidation.

Actinobacillus

These are non-motile, non-branching, Gram-negative, non-acid-fast rods about 1·5 microns in length, sometimes occurring in long chains. One of the members of this group, *Actinobacillus*

lignieresi, causes a granulomatous disease in cattle called actinobacillosis, resembling actinomycosis in several respects. This organism also grows in the tissues in colonies and gives rise to the production of granules which are smaller but more numerous than the granules of *Actinomyces*. Another species, *Actinobacillus actinomycetum comitans*, has been isolated from the lesions caused by *Act. bovis* (p. 588).

Leptotrichia

The members of this genus are entirely saprophytic. They consist of unbranched filaments sometimes provided with septa. *Leptotrichia buccalis* (*Leptothrix buccalis*) is a species often found in the normal mouth. It is Gram-negative and aerobic. It may occasionally be seen in suppurative lesions of the mouth and throat, but that may be only a reflection of its presence in the mouth.

Erysipelothrix

The members of this genus are rod-shaped organisms with a tendency to the formation of long filaments which may show branching. They are non-motile and Gram-negative. No spores are developed. Granules may be seen in the cells. The members are microaerophilic. Usually they are parasitic.

Erysipelothrix Rhusiopathiae. This organism exists in two morphological types, probably smooth and rough. The smooth form shows small, slender, straight or slightly curved rods, 0·8–2·0 microns by 0·3–0·4 micron, occurring singly, in small groups or in short chains. Long chains of bacilli and long interlacing filamentous forms characterise the rough type. The organism is non-motile, non-sporing and Gram-positive. Deeply stained granules may be a prominent feature.

The species is microaerophilic but may grow under aerobic as well as anaerobic conditions. Growth occurs even at room temperature, though the optimum temperature is 37° C. Growth is improved by glucose and to a less extent by blood. The smooth form shows round, convex, small, water-clear colonies with smooth, glistening surface. In gelatin stab culture a line of growth occurs along the needle track, except at the surface, with lateral prolongations radiating from the central line of growth. Broth cultures show uniform turbidity. The colonies of the rough

form are larger and flatter with a dry granular appearance. Growth in liquid media gives rise to fluffy hair-like masses with little or no turbidity. The deep colonies of *E. rhusiopathiae* in ten per cent. horse blood agar show haemolysis.

Of the common sugars, only glucose, lactose, galactose and levulose are fermented with the production of only acid. Indole is negative. Litmus milk is unchanged. Nitrates are reduced. Hydrogen sulphide is formed.

Resistance is slightly higher than in the case of other vegetative bacteria. Moist heat kills the organism in fifteen minutes. The organism is very resistant to preservative processes, like salting, pickling and smoking; in such preserved meat it may survive even up to one to three months. Great resistance is also shown to putrefaction. These are points of practical importance in food industry.

E. rhusiopathiae does not produce any exotoxin. It causes a spontaneous disease, swine erysipelas, in swine. It may also rarely infect sheep and birds. In the infected animals the bacilli are present in the characteristic lesions and in the internal organs, like the spleen and kidneys. Marked septicaemia is present in some cases, when the organism is seen in blood films. Occasionally man may get the infection. Experimentally it is pathogenic for mice, pigeons and rabbits, but not for guinea-pigs. Inoculation into swine reproduces the disease just as it occurs naturally.

The human disease is called erysipeloid. Transmission is through contact with swine, the organism entering through some abrasion in the skin during handling of infected materials, like meat, hides and manure. It may also be noted that the organism is present in the normal hog and has been isolated from the tonsil, intestine and faeces.

Antibodies, like agglutinins, are produced in the infected pig and their appearance is made use of in diagnosis. An antiserum prepared against the organism has some therapeutic and prophylactic value. Active immunisation with attenuated culture, or with virulent culture in conjunction with antiserum, is valuable.

CHAPTER XXXI

BACILLUS—THE ANTHRAX BACILLUS

The family *Bacillaceae* comprise a very large number of species widely distributed in nature, producing endospores, usually Gram-positive and, when motile, the flagella arranged in a peritrichous fashion. On the basis of oxygen requirement, they are classified into two genera, the *Bacillus* or the aerobic forms and *Clostridium* which includes all the anaerobic spore-forming bacilli. Most of the organisms are saprophytic, but a few are pathogenic. The term *Bacillus*, as here used, is in the special generic sense. Any rod-shaped organism may be called a bacillus and often the term is loosely employed in either sense. When used in the generic sense, the word begins with a capital letter.

The *Bacilli* are large, aerobic, spore-bearing organisms, often growing in enormously long chains and forming rhizoid colonies on solid media and loose flake-like masses in liquid media. The sporing does not considerably change the shape of the mother cell. With the exception of the anthrax bacillus and a few others, all are motile by means of peritrichate flagella. *Bacillus anthracis* is the only one that forms capsule. The organisms of this group are generally Gram-positive, displaying varying grades of positivity, but a few are frankly Gram-negative. Almost all the species are non-pathogenic; *B. anthracis* is the only human pathogen. Others are frequently encountered as contaminants in culture media. They are widely distributed in nature, in air, soil, water, milk, dust, wool, faeces and other situations. The Type species is *Bacillus subtilis*. Other well known species are *Bacillus mesentericus*, *Bacillus megatherium*, *Bacillus mycoides* and *Bacillus vulgatus* or the "potato bacillus".

Bacillus Anthracis. The anthrax bacillus has a special historic interest in that it was the first micro-organism to be definitely proved to be causally related to an infectious disease. Although others had seen it previously in the tissues of animals

dying of anthrax, it was Koch (1877) who, by reproducing the disease with a culture of the organism, proved conclusively its aetiological role in anthrax. He also described the organism. Obviously, this discovery of Koch must have contributed not a little to the development of bacteriology. It may also be mentioned that one of the earliest attempts at active immunisation was also against anthrax by Pasteur.

Ecology. The anthrax bacillus causes natural disease in cattle, sheep and other herbivorous animals and man and it is present in the lesions produced. Spores, as they remain viable for a long time, may be found in the soil, dust, wool and other situations.

Morphology and Staining. They are large rods, 3·0–9·0 × 1·5 microns, usually straight with a rectangular appearance. Long chain-formation in culture is a characteristic feature; but what we usually see in tissues and blood are short chains of three or four, end to end pairs and singles. The spores are oval in shape and equatorially placed, not bulging the mother cell. They are not formed in the host body as they require oxygen for development. Germination of the spore is polar or very rarely equatorial. The organism is capsulated in the blood, tissues and media containing serum. The anthrax bacillus is non-motile.

The anthrax bacillus is easily stained by the ordinary coal-tar dyes, but spore and capsule require special methods of staining. Staining with a 1 per cent. watery solution of polychrome methylene blue brings out the capsule well (McFadyean). Quite apart from the presence of the spore, the organism often exhibits uneven staining. This is due to the presence of certain intracellular granules, composed of fat, volutin or glycogen. *B. anthracis* is Gram-positive and non-acid-fast.

Growth Requirements. *B. anthracis* is an aerobe and facultative anaerobe. Molecular oxygen is necessary for sporulation and the optimum temperature for it is 25°–30° C. The optimum temperature for growth is 37° C. with a range of 12°–44° C. The anthrax bacillus does not need any special nutritional factors for growth; it will grow well on all media. The addition of blood does not improve growth.

Cultural Characters. On agar colonies are irregularly round, raised, opaque with irregular, wavy hair-like edge and roughish surface, sometimes referred to as "Medusa head"; the whole

colony consists of long tangled chains, extremely difficult to emulsify. Smooth colonies may appear during subculture. They are relatively avirulent. On gelatin, stab culture gives rise to the so-called "inverted fir tree" growth, *i.e.*, a line of growth along the needle track with long lateral extensions at top which become shorter and shorter with increasing depth. In broth there is no turbidity nor surface growth, but a white flocculent deposit is formed.

Biochemical Reactions. Acid without gas is formed in a few sugars, like glucose, saccharose and maltose; lactose, mannite, dulcite, xylose and rhamnose are not fermented at all. Indole is not formed. Nitrates are not reduced. Little or no hydrogen sulphide is produced. Litmus milk is rendered slightly acid followed by coagulation and peptonisation. The anthrax bacillus liquefies gelatin but slowly as against the rapid liquefaction by the anthracoid bacilli. Coagulated serum is also partially liquefied.

Resistance. Vegetative forms are easily destroyed. Spores are very resistant, withstanding a temperature of 80° C. for half an hour. But they are killed by boiling for ten minutes. While the vegetative forms are killed by the gastric hydrochloric acid, spores escape its destructive action. Of all the chemical germicides, the oxidising ones are more destructive to the spores. Potassium permanganate in 4 per cent. concentration kills spores in fifteen minutes and 3 per cent. hydrogen peroxide in one hour. Desiccation is not destructive to the spores, and when dry, they remain alive for years. Thus, infected wool, hairs, hides and dust may remain infective for many years. This is of great economic importance, for in certain industries, such as the wool and leather industries, the contaminating spores have to be destroyed from raw material before use. Spores also remain viable for many years in the soil, as long as twenty to thirty years.

Variation. When freshly isolated and virulent, the colonies are rough. But after many subcultures, or by attenuation of virulence by artificial methods, moist smooth colonies are formed, consisting of bacilli devoid of capsule and arranged in bundles without the characteristic chain formation of the rough type. Associated with this smooth variation there is also loss of virulence (p. 345).

When the organism is grown for a long time at a higher than optimum temperature, 42°–43° C., the result is an attenuation of

the strain and the appearance of asporogenous variants. The change is a gradual one and intermediate forms with reference to virulence and colony formation are seen. Similar changes are also brought about by prolonged cultivation in the presence of dilute antiseptics. There appears to exist a much greater correlation between virulence and the capacity to form capsule than between virulence and the capacity to form spores. For, both spore-forming avirulent strains and asporogenous virulent strains may be encountered (p. 332).

Antigenic Structure. Two antigenic constituents have been definitely defined—a capsular antigen and a somatic antigen. The former, unlike the capsular antigen of the pneumococcus, consists of a protein-like substance which is most probably a polypeptide. It is a group-specific hapten. The somatic antigen, on the other hand, is a polysaccharide antigen. Each antigen reacts specifically with the corresponding antiserum.

Toxin Production. No soluble toxin is known in the case of *B. anthracis*. Attempts to establish toxicity for the cell substance have not been conclusive. Death is not due merely to mechanical causes, for it occurs even in the absence of great numbers of bacilli. The exact manner of pathogenesis still remains obscure. The virulence of the bacillus is in all likelihood dependent on capsule formation. The anthrax bacillus is highly invasive and the organism is probably protected by its capsule. The capsular substance may have also a specific anti-opsonic effect. Probably for these reasons, septicaemia is very common. In fact, anthrax, as in plague, illustrates strikingly that when the host resistance is feeble, the invading bacillus multiplies unchecked in enormous numbers throughout the body. In anthrax, no doubt, the capillaries are often found to be mechanically blocked by masses of bacilli and death may be occurring also as the result of capillary embolism, especially in the brain.

Pathogenicity. The anthrax bacillus is highly pathogenic to animals and man. It causes spontaneous disease in the former and from them man gets the infection. Of the animals, the herbivorous animals are particularly susceptible. The carnivorous animals are the most resistant, but epizootics among them have been reported. Birds, with the exception of sparrows, are resistant.

Experimentally mice, guinea-pigs and rabbits are easily infected. A single virulent bacillus is said to be enough to kill

a white mouse. Rats are quite resistant, both naturally and experimentally. Subcutaneous inoculation of the organism into a susceptible animal is usually followed by death in two days. Severe inflammatory reaction at the site of inoculation, gelatinous oedema, profound septicaemia and enlarged soft diffluent spleen are characteristic features. The organism is found in large numbers in the local lesions, heart blood and the viscera, particularly the spleen.

Anthrax among animals is present all over the world, but it has greater prevalence in Russia and parts of Asia. The main victims are the herbivorous animals, especially cattle, sheep and goats, but the Algerian sheep is immune to anthrax. Horses and hogs may also be affected. Spread among animals occurs through contaminated pasturage, artificial foodstuffs and rarely by biting flies, like *Stomoxys*, acting as portal carriers. Thus, infection enters mainly through the alimentary tract. The disease is essentially septicaemic; the bacilli are present in large numbers in the blood and are discharged in the saliva, urine and faeces. Extensive contamination of pasturage is therefore inevitable. Death occurs in two or three days. Post-mortem examination reveals the presence of a dense bacillary population of the blood and internal organs, especially the spleen which is enlarged, dark red, soft and diffluent. It is this last point that has given the name "splenic fever" to this disease. Masses of bacilli are found to clog the capillaries. The death rate varies between 75 and 100 per cent.

As indicated above, man gets the infection from some animal source, directly or indirectly. Anthrax is an occupational disease. In the non-industrial type, shepherds, farmers, veterinary surgeons, butchers and others coming in contact with infected carcases may themselves get infected. Another rare mode of transport of the infection is through infected shaving brush. In the industrial type the handling of wool, bristles, hides and skins, in such industries as tanning, harness making, etc., may transmit the infection to man. In these materials the anthrax spores survive for very long periods. There are three clinical types, the cutaneous, pulmonary and alimentary, as determined by the portal of entry.

Infection through the skin causes the cutaneous type, called malignant pustule. This is the usual form in the non-industria

type. Pus formation is not a conspicuous feature of malignant pustule which is also not very malignant. Hence this term suffers from descriptive inaccuracy. An indurated papule with central necrosis and surrounding vesicles forms the main feature; there may be oedema about the lesion. In fatal cases septicaemia supervenes. The death rate is relatively low, 10–20 per cent. When the infection enters through the respiratory tract (with dust containing spores), pulmonary anthrax, called wool sorter's disease, results. This is the usual industrial type. The seat of primary lesion is in the trachea or bronchi. It is really a malignant form with septicaemia and generalised infection. The fatality rate is practically 100 per cent. The intestinal type, which is the usual form in cattle and sheep, is very rare in man, but it is practically always fatal after a short septicaemic course. Characteristic primary lesions of the intestinal mucosa are seen at autopsy. Obviously, consumption of food infected with spores is the cause.

Immunity. The basis of the natural resistance against anthrax is obscure. The production of antibodies against the anthrax bacillus is not very conspicuous. In the artificial immunisation of horses it is slow and seldom attains a high degree. Asses respond better and antisera are now prepared from them. Inoculation with antisera confers some degree of protection. The mechanism is probably phagocytic, in which the bacilli are sensitised by the antibodies. The presence of antibodies formed against the capsular substance may be important in determining the potency of an antiserum. Immunity may follow recovery from the disease, but it is of short duration and subsequent attacks may occur.

Diagnosis. Anthrax is a notifiable disease in some countries and post mortem of the infected animal is illegal. The carcase is buried with lime well below the surface or incinerated. For diagnostic purposes an ear is cut off and sent to the laboratory. The procedure adopted in the diagnosis of human anthrax is also applicable in the diagnosis of animal anthrax.

In the case of human anthrax the vesicular fluid in the skin type, the sputum in wool sorter's disease or blood, if septicaemia has developed, are the materials to be examined. Smears are made from these, stained by the Gram's method and examined for the presence of the bacillus. A positive result should not be taken as conclusive except in the case of malignant pustule.

The isolation of the organism followed by its identification by the serological and pathogenicity tests should then be proceeded with before a positive report is given. It is very important to do the pathogenicity test on the isolated organism as there can be many morphologically identical, but harmless, organisms present confusing the issue. It is done in mice or guinea-pigs. Instead of the isolated culture, straight inoculation subcutaneously of the infected material may also be resorted to. If the material is contaminated with other organisms, as it often will be, it is heated at 80° C. for 20 minutes and inoculated. The infected and contaminated material may also be rubbed into the scarified skin; this however is not very satisfactory as the animal may die of other infection. In a positive case the animal develops anthrax and dies in two to three days, exhibiting the characteristic features mentioned above.

Ascoli's *thermoprecipitin test* is often valuable. This is done by extracting the infected material and employing the extract as antigen in a precipitation test with the immune serum. Two grams of the suspected tissue are macerated in 5 c.c. of sterile normal saline, boiled for 5 minutes and filtered. The filtrate is then slightly acidified with dilute acetic acid. A column of it is introduced into a narrow test tube and the specific serum run along the wall of the tube. The formation of a white ring at the surface of contact indicates a positive result. Other serological reactions are of no help in diagnosis.

The examination of shaving brush for the presence of anthrax spores may sometimes be called for. The bristles are cut into small bits with scissors and soaked in a fairly large volume of sterile salt solution or in a 3–5 per cent. solution of caustic potash. The container is shaken vigorously for some time and the pieces allowed to settle. The supernatant fluid is decanted and centrifuged. The sediment is then resuspended in a small volume of sterile saline and heated at 80° C. for thirty minutes, in order to destroy the vegetative forms that may be present. Cultures are then made from this and further measures of identification are adopted as detailed above. The same procedure is followed for testing suspected wool and other materials.

Treatment. Sclavo's serum prepared from the ass is useful. It is given in 50–100 c.c. doses intravenously or intramuscularly and repeated if necessary. The best results are obtained in the

cutaneous type. How it acts, is not known. The serum does not contain any antitoxin or bactericidal substances and exhibits only a very low agglutinin content.

Another usual, but non-specific, remedy is arsenic (N.A.B.). The results of treatment with the sulphonamides and penicillin are inconclusive.

Prophylaxis. This may be resolved into general and special. General prophylaxis consists in the proper disposal of the dead animal (deep burial surrounded by lime or incineration) and the prevention of infection by legislative, educative and other measures. The disease should be made notifiable and the post-mortem examination of dead animals illegal. By the strict enforcement of the rules of industrial hygiene, the incidence of anthrax in factories can be greatly reduced or even abolished. Shaving brush from suspected sources should be adequately sterilised. The specific measures available at present are not applicable to man but only to animals. Vaccination with killed anthrax bacilli has no immunising value. Hence, live attenuated vaccine is used to induce active immunity. Pasteur's attenuated vaccine is the one in use. Attenuation is effected by growing the organism at a temperature between 42° and 43° C. It is true that there is no control over the extent of attenuation. The sporogenic capacity is also lost during the process. A 21-day attenuated culture is employed in 1 c.c. quantity for the first dose. After 10–12 days a second dose of less attenuated culture, 12-day attenuated, is given. Immunity so engendered may last for one to two years. This procedure is quite useful but does not protect all animals. Pasteur's high results have not been obtained by others. A combined vaccine and serum, serovaccine, is also employed in some places for immunisation.

Related Species. Organisms closely resembling the anthrax bacillus have been isolated from various situations. Such organisms have been frequently referred to as *Bacillus anthracoides* or *Bacillus pseudoanthracis*. The well known members are *B. subtilis*, *B. megatherium*, *B. mesentericus*, *B. mycoides* and *B. vulgatus*. They may be mistaken for the anthrax bacillus and so it is necessary to know the main differential features between them.

The anthrax bacillus is non-motile, while most of the saprophytic members are motile. The anthrax bacillus is capsulated.

whereas capsulation is not a feature of most of the other members. While the anthrax bacillus grows in long chains and in the inverted fir tree fashion in gelatin stab culture, the saprophytes do not exhibit these features. The anthrax bacillus liquefies gelatin slowly, while the others do it rapidly. *B. anthracis* alone is pathogenic to laboratory animals. But it may be remembered that if these animals are injected with any of the anthracoid bacilli in massive doses, it may cause inflammation and oedema, though of a mild grade, with multiplication of bacilli, and their finding, in the tissues. Ascoli's precipitation test is positive in the case of the anthrax bacillus but negative in the case of others.

B. subtilis, or the hay bacillus, is found in hay, dust, milk, soil, water. It is 3–4 μ long, with rounded ends and occurring in singles or short chains. It is actively motile by means of peritrichate flagella. It is non-capsulated. The spores are oval and central or subterminal in position. Slight swelling of the rod may be noticed on maturation of the spore. The organism is Gram-positive. It grows freely on ordinary culture media; in fact, it is a frequent contaminant of these. Acid is produced in glucose, saccharose, maltose and salicin. No indole is formed.

B. subtilis is generally non-pathogenic. It has been reported from cases of conjunctivitis and other inflammatory conditions of the eye. In run down conditions it may even invade the blood stream.

CHAPTER XXXII

CLOSTRIDIUM—THE SPORE-BEARING ANAEROBES

This genus includes all the anaerobic or microaerophilic bacilli forming endospores which alter the shape of the parent cell. Many of the species are motile by means of peritrichate flagella. A few are capsulated. All are Gram-positive.

Members of this genus are widely distributed in nature; they occur in the soil and the intestinal canal of animals and man. By faecal and soil pollution their spores get into dust, sewage, water and milk. There are two possibilities with regard to their permanent abode: they may be permanent denizens of the soil and their presence in the intestine is the result of consuming contaminated food and drink; alternately, their natural habitat may be the intestinal tract of animals and man and their presence in the soil is but a reflection of its faecal contamination. The balance of existing evidence is in favour of the former view.

Clostridium contains most of the important putrefying agents in nature, which lead a saprophytic existence, breaking down complex dead organic bodies into simple compounds readily utilisable by the higher forms of life. Thus, they play an important role in the economy of nature. Though saprophytic, many species are causally related to several human and animal diseases, producing very powerful soluble toxins. Such are *Clostridium tetani*, *Clostridium septicum*, *Clostridium welchii*, *Clostridium novyi* (*oedematiens*), *Clostridium histolyticum*, *Clostridium chauvoei* and *Clostridium botulinum*. Besides these, scores of saprophytes have been described, but most of them have been only incompletely studied.

In general, their power of invading the body is very limited and certain conditions, like the presence of traumatic injury, are essential before infection can be established. *Cl. botulinum* is altogether incapable of causing infection. *Cl. tetani*, though able to set up local infection, has no invasive powers. Without some traumatic injury or exposed raw focus, the gas gangrene

bacilli are unable to gain a foothold into the body, but, when once they gain entry and establish infection, they display varying degrees of invasive powers.

Classification. These organisms had been recognised by the early workers. But owing to the inherent difficulties of cultivating and isolating them in pure culture, their studies were very incomplete and the results inaccurate. Inevitably, therefore, their investigations did not lead to the development of a correct nomenclature or sound classification. During the war of 1914–18, considerable advances were made in our knowledge regarding these organisms. As a result, these organisms were brought under some sort of stable classification. As more precise knowledge accrues it may become necessary to alter this.

TABLE XIX

Classification of the Important Species of Clostridia

Position of spores	Both proteolytic and saccharolytic		Slightly proteolytic, not saccharolytic	Saccharolytic, not proteolytic
	Predominantly proteolytic	Predominantly saccharolytic		
Central or subterminal	*Cl. sporogenes* *Cl. histolyticum* *Cl. botulinum*	*Cl. welchii* *Cl. septicum* *Cl. novyi* (oedematiens) *Cl. chauvoei*		*Cl. butyricum* *Cl. fallax*
Terminal and spherical			*Cl. tetani*	*Cl. tetanomorphum*

On the basis of their biochemical activities, they are broadly classified as proteolytic and saccharolytic. Further classification is made on the basis of the shape and position of the spore in the cell. All the human pathogens, other than *Cl. tetani* and *Cl. botulinum* the toxin of which causes one type of food intoxication, come under the gas gangrene group. The proteolytic type attacks protein, digesting it through the agency of proteolytic ferments. As the result of digestion, meat in the meat medium and flesh in the lesions are decomposed and blackened with the formation of foul-smelling compounds containing

sulphur. The process of protein digestion is carried further to the production of amino acids, and tyrosine crystals frequently appear as feathery processes round the meat particles of the medium. The saccharolytic type grows luxuriantly and rapidly in carbohydrate media, producing large amounts of volatile acids and abundant gas. When the medium contains both carbohydrates and protein as in the meat medium, meat is not digested but is coloured pink by the acid. The proteolytic and saccharolytic properties, however, are not mutually exclusive. While the proteolytic ones are predominantly proteolytic, they may also possess saccharolytic properties in a less degree and the saccharolytic ones, on the other hand, may also be weakly proteolytic; for example, *Cl. welchii*, a saccharolytic member, produces during growth small amounts of amino acids in addition to acid and gas.

Clostridium Tetani. The tetanus bacillus was discovered by Nicolaier in 1884 in materials obtained from mice and other animals that had died of experimental inoculation with soil. But it was not till 1889, when Kitasato succeeded in isolating it and reproducing the disease with pure culture in experimental animals, that its causal role in tetanus was established. The same worker also demonstrated that the tetanus bacillus did not enter the blood stream but remained confined to the primary lesion from which it set up the characteristic intoxication known as tetanus. It was again the epoch-making work of Behring and Kitasato (1890) with tetanus and diphtheria antitoxins that laid the foundation of serum therapy.

Ecology. The organism is present in the soil, especially manured cultivated soil, and in the alimentary canal of certain herbivorous animals and man. In man it is probably an organism of passage. Whether the organism thrives as a saprophyte in the soil or whether its primary habitat is the intestine of animals, is still unsettled.

Morphology and Staining. They are rods, 0.4–0·6 × 4.0–8.0 microns, straight, round ended; usually arranged singly, but pairs, chains, shorter forms and long filamentous forms may all be seen. The tetanus bacillus is sluggishly motile with 20–30 peritrichous flagella. The spores are spherical, terminal and three or four times broader than the mother cell, the whole structure thus presenting the characteristic drumstick appearance. The organism is non-capsulated, Gram-positive and non-acid-fast.

Growth Requirements. *Cl. tetani* is a strict anaerobe. The optimum temperature is 37° C. and the range is 14°–43° C. Spore-formation is delayed for a week or more at room temperature, whereas at 37° C. it commences in two days. The tetanus bacillus grows fairly well on the usual laboratory media; blood as well as serum improves growth. Its nutritional requirements are rather complex, requiring many amino acids and vitamins when grown on synthetic media.

Cultural Characters. *Cl. tetani* grows on ordinary media provided the suitable gas phase is ensured. But it grows rather slowly and takes about 3–4 days for good growth. On agar plate irregular, round, greyish colonies are formed with ill-defined edge, showing filamentous curly projections. The surface is granular and shows a brownish, raised central area and a surrounding thin colourless periphery. The colonies are butyrous in consistence and emulsify easily. If the water of condensation of an agar slope is inocualted, the organism grows up the slope spreading almost to the top in 24 hours by means of feathery or fern like offshoots. On this character depends Fildes' method of isolation of the organism. On agar stab culture, except near the surface, growth occurs as a white streak along the needle track. Lateral branches then develop from the central stem. They are shorter towards the surface, giving the so-called "fir tree" appearance. The same occurs in gelatin stab, but in a few days the fir tree appearance disappears owing to the liquefaction of gelatin. An agar shake culture shows the upper one inch entirely devoid of any growth. Below this, scattered fluffy colonies are formed, more numerous in the deeper reaches. If glucose also had been added to the agar, as is usually done, slight amounts of gas are formed, which disrupt the medium. Growth in broth renders it slightly turbid and gives rise to the formation of a moderate amount of granular deposit. In the cooked meat medium, the organism grows well and may effect in course of time very slight darkening of meat.

Biochemical Reactions. The tetanus bacillus ferments no carbohydrate and it has only weak proteolytic activity. Indole is formed. Litmus milk is not changed or very slowly changed with slight precipitation of casein. Nitrates are not reduced; hydrogen sulphide is formed. A haemolysin is produced.

Resistance. Vegetative forms are readily destroyed by adverse agencies, but spores are very resistant, withstanding the temperature of boiling water for 15–90 minutes. Moist heat at 120° C. destroys them in 20 minutes. Tetanus spores withstand dry heat at 150° C. for one hour. Five per cent. phenol destroys them in fifteen hours and 1 : 1,000 perchloride in two to three hours.

Antigenic Structure. *Cl. tetani* is antigenically heterogeneous. On the basis of agglutination reaction, a number of serologically different types, at least ten, have been defined. The toxins produced by these various types are immunologically identical and the toxin of any type will be specifically neutralised by antitoxin prepared against any other type. But agglutinins and opsonins, specific to each type, are present in antibacterial sera. Types I and III are the commonest in Britian; there is no definite knowledge about the type prevalence in this country.

Toxin Production. *Cl. tetani* produces a powerful soluble toxin. It consists of two moities: tetanolysin and tetanospasmin. It is on the latter that the pathogenicity of the organism depends; the former does not seem to play any significant part in pathogenesis. There are atoxic and avirulent strains of tetanus bacilli. Such strains may produce the haemolysin but not the tetanospasmin. As noted above, different types of tetanus bacilli produce the same kind of toxin. The tetanus toxin is highly antigenic and gives rise to an antitoxic serum of high potency.

The toxin is prepared by growing the organism in broth for 5–14 days and then filtering off the bacilli by the Berkefeld, Chamberland or any other bacterial filter. It is a very virulent poison. Even a dose of 0·0001 ml. administered parenterally will kill a mouse, the animal dying with characteristic symptoms. Weight by weight horses are twelve times and guinea-pigs six times more susceptible than mice. The toxin is not toxic on feeding. Apparently, it is not absorbed from the gut; it may be also destroyed by the gut contents; the proteolytic enzymes readily destroy it. It is highly susceptible to heat, light, acids and alkalies. Heat at 65° C. destroys it in five minutes. If dried and preserved in the vacuum and in the dark at 5° C., it will keep unchanged for two years or more. The tetanus toxin is rendered atoxic by the action of formaldehyde. Toxin mixed with 0·3–0·4 per cent. formalin and incubated at 37° C. for several weeks

becomes non-toxic, 5–10 c.c. of the product proving harmless to the guinea-pig. This plain toxoid is precipitated with 2 per cent. alum and the precipitate is washed and resuspended in sterile normal saline. This is alum precipitated toxoid.

Pathogenicity. The organism is naturally pathogenic to man and animals and experimentally to mice, guinea-pigs and rabbits. Birds are resistant; their brain cells are unable to fix the toxin. The clinical picture of the disease in animals is similar to that in man. Neither the infected man nor animal is usually concerned with the transport of the infection.

Tetanus. Tetanus bacilli and their spores occur widely distributed in nature and yet the incidence of the disease is relatively low. Though the disease is universal in distribution, it is more common in the tropics. It is through the introduction of the spore form into the tissue that infection results. Though transmission may take place in a variety of ways, the chief method is through contamination of wounds or raw surfaces with spore-containing soil. Other methods are through administration of infected gelatin, use of contaminated catgut, drugs like quinine or vaccine lymph, as a complication in septic abortion or in otitis media or through the umbilical cord in the infant. Though the tetanus bacillus is often found in the human intestine, tetanus following surgical operations involving the gut is exceedingly rare.

The mere implantation of spores is not enough to cause the disease. As in gas gangrene, spores washed free of toxin when introduced into living tissue do not sprout; probably the oxidation-reduction potential of the intact living tissue is too high to permit the sprouting of the tetanus spores. They are engulfed by the phagocytes and taken away to other parts of the body. Such spores may remain alive for a long time. With the advent of favourable circumstances, such as trauma or operation causing death of tissue or the occurrence of haemorrhage and the clotting of blood or the introduction of irritant chemicals, the spores are carried to such areas of low oxidation-reduction potential, where they germinate and the organism rapidly multiplies. In clean aseptic wounds, again, spores are unable to germinate. Sometimes a wound, though infected with spores, may heal without tetanus developing if it is clean. But the infection lies dormant in the body. And after several weeks or months tetanus might

develop suddenly after an operation, perhaps on some other part of the body. Circumstances like these probably explain the occurrence of what is called idiopathic tetanus. All these observations tend to show that infection does not always lead to the immediate development of tetanus. Aside from the implantation of the spores, certain conditions are also necessary to determine infection, the most important being the presence of a suitable gas phase at the site of infection, without which spores will not sprout.

As mentioned above, the common method of infection is through wounds contaminated with soil, such as compound fracture, penetrating wounds, war wounds and the like. Soil, small splinters, pieces of rags and similar materials cause irritation and local devitalisation of tissues. These dead tissues form the ideal pabulam, affording the low oxygen potential necessary for the germination of spores and the multiplication of the microbe. Badly lacerated wounds are specially suitable for this process to take place. The low oxygen tension of the dead and dying tissues is further reduced by the aerobes not infrequently associated with tetanus infection and found flourishing in such wounds. Among the poorer classes of this country the first aid, or even all the aid at times, is rendered often by the application of cow dung, soil or dry rags, quite manifestly a very dangerous practice.

Howsoever the infection might be transmitted, the implanted spores sprout when the conditions are favourable and the organism multiplies rapidly, liberating toxin. Remarkably few organisms, it has been noted, are enough to start disease. Other associated organisms may also liberate their toxic factors. The infection is strictly confined to the primary focus and tetanus is essentially an intoxication. Toxin that is formed and set free is absorbed and taken finally to the central nervous system on which it selectively acts, giving rise to the characteristic symptom complex of the disease.

How exactly tetanus toxin is absorbed from the site of infection and transmitted to the central nervous system, is by no means definitely known. Several routes are possible. It may be absorbed by the end-organs of the motor nerves and carried upwards by the axis cylinder process to the focal anterior horn cells and thence disseminated to other homologous cells. And

a small fraction may also gain entrance into the circulation to be likewise dealt with by the motor end-plates in other parts of the body. This mode of absorption by the end-plates seems to be the most probable way. A cut motor nerve end does not seem to absorb the toxin. The intact sensory nerve also is not capable of absorbing and carrying up the toxin. According to this view, the time taken by the toxin to travel up the spinal centres represents most of the incubation period. A second possible route is via the lymphatics into the circulation, the blood supply finally delivering the toxin into the susceptible brain tissue. Suggestion has also been made that the toxin may be absorbed into the endoneural and perineural lymphatics and carried to the subarachnoid cavity. But there is no evidence that these lymphatics drain into the subarachnoid cavity.

Tetanus toxin has a remarkable affinity for the cells of the central nervous system, as proved by the experiments of Wassermann and Takkaki who showed that toxin mixed with brain matter can be inoculated into a susceptible animal without causing any untoward effect. Their experiments also demonstrated the capacity of the brain cells to fix the toxin in vitro. The nerve cells of the susceptible animals fix the tetanus toxin. Only after such intracellular fixation can the toxin act. The nervous system of the naturally immune animals and birds, on the other hand, lack the capacity to fix the tetanus toxin. In them the circulating tetanus toxin is destroyed by the tissues. In the susceptible species the fixed toxin acts upon the nerve cells, setting up irritation and eventually death. Hence, when once the toxin has been fixed in the cell, the circulating antitoxin cannot readily neutralise it. It is manifest, therefore, why the administration of antitoxin is not of much value when symptoms have declared.

The position and nature of the wound are also of some importance in determining the incubation period and clinical severity of the disease. The nearer the wound to the head, the shorter the incubation period and severer the disease. The greater the laceration of the parts, the more serious the disease. The incubation period of tetanus is 3–21 days, may be longer up to fifty or more days. The mortality rate is very high, ranging from 80–90 per cent.; it exhibits an inverse relationship to the incubation time, the longer the incubation period the greater the chances of survival.

Immunity. The immunity conferred by one attack is low and of short duration. Subsequent attacks have been reported. Active immunisation is relatively easy to induce. There is little evidence to show that man or animals may become actively immunised as a result of an intestinal carrier state.

Diagnosis. It is by no means easy to establish a bacteriological diagnosis of tetanus. The number of organisms present in the lesion may be very few; the organism is strictly anaerobic and practically always there are concomitant organisms, both aerobic and anaerobic, spore-bearers and non-spore-bearers. All these factors render cultivation and isolation laborious and prolonged. The isolation of the organism is also rendered extremely difficult from the fact that the spores of different species tend to stick together.

Microscopic examination of materials obtained from the wound may be attempted. The discovering of tetanus bacilli in the wound is but rarely successful. It should be remembered that the presence of the characteristic, drumstick-shaped, spore-bearing bacilli does not establish the diagnosis, as other organisms morphologically similar but non-pathogenic, like *Cl. tetanomorphum*, may be present. Hence, further investigations are essential.

Owing to the above mentioned complexities, the isolation of a pure culture of the organism is an unusually difficult piece of work. For cultivation any of the anaerobic methods may be employed. Before inoculation the material is suspended in some sterile saline, heated at 80° C. for 10–15 minutes to destroy all vegetative forms and inoculated on agar plate for isolation. Alternately, the untreated material is inoculated into Robertson's cooked meat medium and after 2–3 days of growth, the culture is heated at 80° C. for 20–30 minutes and plated for isolation. Repeated plating may be necessary. Fildes' method is relatively simple and yields satisfactory results (see above). The identification is then made by the study of the biochemical charecters and by serological methods. Even this may not be conclusive and a virulence test may become necessary to establish the diagnosis.

Virulence Test. The virulence test is usually done on mice or guinea-pigs. Either a suspension of the material removed from the primary focus or a pure culture after isolation is used

for inoculation. Where possible, the pure culture method is to be preferred, as it is more reliable. Though the symptoms of tetanus in animals are characteristic, it is more trustworthy to include a control. The control animal receives previously a protective dose of tetanus antitoxin. The suspended material is inoculated subcutaneously into both animals. The unprotected animal develops the disease showing characteristic spasms and dies in about one to four days, while the protected one remains unaffected.

Treatment. The therapeutic value of tetanus antiserum may not be great. The antitoxin cannot repair the damage done by toxin. Direct contact is necessary for the antitoxic serum to neutralise the toxin. While, therefore, the antitoxin will neutralise any circulating toxin, it will not touch the cell-fixed toxin which is really the disease-causing portion. Though the nervous tissue of mammals has a great affinity for toxin, it has not the same for antitoxin and so the latter does not find easy access into the cells wherein the toxin has already been lodged. Hence the importance of giving antitoxin as early as possible and its futility when once symptoms have developed. Every hour of delay lessens the chances of recovery. Antitoxin has no effect on the organism or its spore.

Though there is no definite evidence to prove the curative usefulness of antitoxic serum, it has, nevertheless, to be given at whatever stage the case is seen. If the case is serious, large doses have to be given through all the three routes—intraspinal, intravenous and intramuscular, and repeated if necessary. The dosage depends upon the condition of the patient. Children require about the same dosage as adults. A dose of 20,000–40,000 international units may be employed, depending upon the seriousness of the case. Large doses, up to 200,000 units, given intraspinally, have been claimed to yield greater success.

Antitoxin is prepared by immunising the horse first with toxoid and later with toxin. It is standardised with reference to the international unit of antitoxin. This is five times the minimum quantity of antitoxic serum required to preserve the life of a guinea-pig of 350 grams weight for 96 hours when mixed with 100 M.L.D. of toxin and injected subcutaneously. The procedure is the same as in the assaying of diphtheria antitoxin (p. 528). The antitoxin is contained in the pseudoglobulin

fraction and is concentrated as in the case of diphtheria antitoxin (p. 530).

Prophylaxis. Antitoxic serum is of undoubted value in prophylaxis, as proved during the 1914–18 war. The disease may be averted or, at least, the incubation period may be prolonged and the disease rendered very mild and more amenable to treatment. A dose of 3,000 units should be given intramuscularly immediately the patient is seen and before any surgical measures are started. It should be repeated once or twice at weekly intervals if the wound is not healing properly. A portion of the serum may with advantage be injected around the wound. Surgical care of the wound, including full excision of all dead tissue, is very important. Cauterisation should never be done. As a precautionary measure, 15,000 units of antitoxin may be given two days before certain operations like the removal of foreign bodies or sequestra. Passive immunity wears out in two to three weeks. Hence, it should be reinforced by active immunisation. For active immunisation against tetanus formolised alum toxoid is now used as it is superior to plain toxoid in its immunising quality. Two doses of 1 c.c. each, administered intramuscularly at an interval of 6–8 weeks, are followed by better results than three doses of plain toxoid. This is reinforced by one more dose after about nine months to a year. The immunity developed does not last longer than one or two years. Mass immunisation against tetanus may not find a place in civil practice, but in the military it is compulsory. There is now a tendency in civil practice for the combined immunisation with tetanus and diphtheria toxoids.

Clostridium Welchii (Clostridium perfringens). Though first cultivated by Achelme, the full description of the bacillus was given by Welch and Nuttall in 1892, who isolated it from the organs of a cadaver and called it *Bacillus aerogenes capsulatus*. It is the commonest cause of gas gangrene.

Habitat. *Cl. welchii* is found in the soil, sewage, water, milk and dust. It is normally present in the large intestine of man and animals. • Ante-mortem invasion of the viscera and blood by this bacillus is a common occurrence. The organism is present in the natural lesions in man and animals.

Morphology. *Cl. welchii* is a short thick bacillus, 4–8 microns long and 1·0–1·5 microns in diameter. It has square or round

ends and the arrangement is in singles, pairs or less frequently in short chains. Spores are oval and subterminal. Sporulation is delayed, especially in the presence of fermentable carbohydrates. The organism is capsulated in tissues. It is non-motile and Gram-positive.

Growth Requirements. *Cl. welchii* is a fairly strict anaerobe, but least strict of all the gas gangrene bacteria. The optimum temperature is 37° C. It grows best on media containing fermentable sugars, but in them it dies out soon due to the destructive effect of acids on the vegetative forms and to the inhibition of sporulation in such environment.

Culture. It grows readily on ordinary media, provided anaerobic methods are followed. On agar two types of surface colonies may be seen, one large, round, smooth, regular, opaque discs, and the other, less common, presenting an opaque centre and radially striated transparent periphery. In glucose agar shake culture the colonies are biconvex and opaque with entire edge. The medium is broken up with abundant gas production. The colonies on blood agar are surrounded by a zone of beta haemolysis. Broth culture gives rise to a uniform turbidity which soon clears up, leaving a viscid sediment.

Biochemical Activity. *Cl. welchii* is actively saccharolytic, forming acid and gas in a number of sugars, such as glucose, saccharose, maltose, lactose and occasionally salicin. In the cooked meat medium, the meat is turned pink but not digested. Milk is coagulated with the production of profuse gas which breaks up the clot into minute fragments, giving rise to the characteristic "stormy fermentation". Indole is not formed. Hydrogen sulphide is produced. Gelatin is liquefied but not usually coagulated serum or egg. The organism forms a soluble haemolysin.

Resistance. The vegetative forms are easily destroyed, but spores are resistant. The organism dies off quickly in acid media.

Antigenic Structure. *Cl. welchii* does not constitute a serologically homogeneous group. Including strains isolated from the lower animals, four serologically different types, A, B, C, D, have been described. The strains occurring normally in the alimentary tract of man and animals and which are responsible for gas gangrene, constitute Type A. Types B, C and D contain strains, closely resembling Type A, that are isolated

from natural diseases of lower animals, Type B from lamb dysentery, Type C from a disease of sheep known as "struck" and Type D, the organism that is associated with enterotoxaemia of sheep. There is considerable sharing of antigen among these types and so cross reactions are common. Further, these types appear to be again divisible into subtypes.

Toxin Production. The members of this species produce an extracellular toxin. It does not seem to be an immunologically simple substance. On the basis of neutralisation tests on experimental animals, usually mice, seven serologically distinct toxins, named α, β, γ, etc., have been identified. Each type of the organism may produce two or more, up to five, of these toxins. The action of the toxins may be lethal, necrotic or haemolytic, any one toxic entity exhibiting any or a combination of these effects. For example, the α toxin is haemolytic, lethal to mice on intravenous inoculation and produces necrosis when injected intradermally into guinea-pigs and rabbits, whereas the γ toxin is only lethal to mice. The production of these toxins is, however, largely dependent on the conditions of growth.

The gas gangrene type, Type A, produces at least three toxic entities, the α toxin being the most important. A culture filtrate is haemolytic and lethal and also displays a local necrotising action on muscle tissue. Its lethal dose for a mouse is about 0·25 cubic centimetre. *Cl. welchii* also produces hyaluronidase.

A toxic filtrate containing the α toxin when mixed with human serum gives rise to an opalescence. The reaction is known as the *Nagler reaction*. The α toxin appears to be an enzyme, lecithinase, or at least it has this property, and the Nagler reaction depends upon the enzymic splitting of the lipoprotein complex of serum. This reaction is useful in the identification of *Cl. welchii*.

Pathogenicity. *Cl. welchii* is the chief agent in the production of gas gangrene. It is found either alone or, more commonly, in association with other anaerobes. It may also cause enteritis, appendicitis, peurperal fever, septic abortion and urinary and gall-bladder infections. It has been isolated from the blood during life in certain septic conditions. In certain animals, like sheep and cattle, it causes spontaneous disease. Experimentally pigeons, mice and guinea-pigs are susceptible, pigeons being the most. In the subcutaneous tissue, about the site of inoculation

a spreading inflammatory oedema with profuse gas production develops. The organism may invade the blood stream. The animal dies in twenty-four hours or after a longer interval, depending upon the virulence of the strain. Autopsy reveals extensive necrosis of the underlying muscles which are friable and pink.

Diagnosis. The diagnosis of gas gangrene is dealt with separately. The diagnosis of other conditions, such as puerperal fever, due to *Cl. welchii* is made on the same lines as in gas gangrene. Uterine discharge, blood and urine should be examined in puerperal fever. It may be borne in mind that anaerobic streptococci may also be present in materials from this condition.

Clostridium Septicum. This is one of the anaerobes causing gas gangrene. Its habitat is chiefly the soil.

Morphology. This species consists of large rods 0·6–0·8 by 3·0–8·0 microns with rounded ends. The occurrence of long chains and filamentous forms on the visceral surfaces in guinea-pigs dead of this infection is very characteristic. Peculiar large swollen forms resembling lemon are also seen in tissues—"citron bodies". Pleomorphism is a marked feature in agar culture. The organism is motile with 4–16 peritrichous flagella. It is not capsulated. It is Gram-positive. Spores are oval, subterminal and swelling the mother cell.

Cultivation. *Cl. septicum* is a strict anaerobe. The optimum temperature is 37° C.

On agar, surface colonies are small and transparent having variable shape. In deep glucose agar delicate, arborescent, sometimes opaque colonies are formed with plenty of gas production. In broth growth gives rise to slight diffuse turbidity followed by clearing.

Biochemical Characters. Glucose, maltose, salicin and lactose are fermented with the production of acid and gas, but not saccharose. In milk slight acidity is produced, which may be followed by clotting. Meat in the meat medium is reddened but not digested. Indole is not formed. Hydrogen sulphide is produced. Gelatin is liquefied but not coagulated serum. A haemolysin is formed.

Toxin Production and Pathogenicity. A powerful exotoxin is generated by this clostridium. Its M.L.D. for mice is about 0·005 cubic centimetre. *Cl. septicum* forms also a fibrinolysin.

The toxin is antigenic and an antitoxin is prepared in the horse. *Cl. septicum* is immunologically related to *Cl. chauvoei.*

Cl. septicum is one of those organisms that cause gas gangrene. Certain natural diseases are caused also in sheep and cattle. Experimentally it is pathogenic for the guinea-pig, rabbit, mouse and pigeon. Inflammatory and haemorrhagic oedema with a slight amount of gas production follows subcutaneous inoculation. The organism may invade the blood stream, producing a septicaemia which is usually fatal in twenty-four to forty-eight hours. After death long filamentous forms and "citron bodies" are noteworthy findings, at the site of injection and in the peritoneal surface of the liver.

Clostridium Novyi (Clostridium Oedematiens). This is the third important gas gangrene organism. It lives chiefly in the soil.

Morphology. It is somewhat like *Cl. welchii,* but more slender with a size of 0·8–0·9 by 2·5–5·0 microns. It occurs singly and in chains. In tissues shorter forms predominate. Though provided with twenty or more peritrichous flagella, motility is sluggish and is observed only under strict anaerobic conditions. It is non-capsulated. Large, oval, central or subterminal spores swell and distort the rods. It is Gram-positive.

Cultivation. It is a strict anaerobe. The optimum temperature is 37° C. It grows best in the presence of a fermentable sugar.

On agar are formed transparent flat colonies which tend to spread. The centre may be often darker than the periphery. The deep glucose agar colonies usually resemble snowflakes, sometimes showing an opaque brownish centre. The medium is disrupted with gas. Broth cultures are slightly turbid with flocculent sediment.

Biochemical Characters. Acid and gas are formed in glucose and maltose but not in saccharose, lactose and salicin. Acid is formed slowly in litmus milk but not clot. Meat in meat medium is reddened but not digested. Indole is not formed, nitrates are not reduced, but hydrogen peroxide is produced. Gelatin is liquefied but not coagulated serum or egg. A haemolysin is formed.

Toxin Production and Pathogenicity. It forms a powerful extracellular toxin, almost comparable in virulence to the toxins of the diphtheria and tetanus bacilli. It is powerfully antigenic

and an antitoxin is prepared in the horse. Novy's bacillus also produces a fibrinolysin.

The organism appears to be specially responsible for the more toxic forms of gas gangrene. But it is also the rarest. When alone it causes less tissue damage than either *Cl. welchii* or *Cl. septicum.* It causes massive, gelatinous oedema without the profuse gas formation of *Cl. welchii* or the haemorrhagic necrosis of *Cl. septicum.* It does not usually invade the blood stream. Novy's bacillus causes natural disease in cattle, horses and hogs. Experimentally guinea-pigs, rabbits, mice and piegons are susceptible. Inoculated intramuscularly into guinea-pigs, it gives rise to a spreading non-haemorrhagic gelatinous oedema, ending fatally in twenty-four to forty-eight hours.

Gas Gangrene. After the advent of antiseptic surgery, the incidence of gas gangrene has been considerably reduced in civil life. But conditions during war remain particularly favourable for its extensive occurrence. In fact, it was the vast opportunities offered during the War of 1914–18 that enabled the first thorough study of the aetiology of gas gangrene.

The disease is caused, together or rarely severally, by the three anaerobic species *Cl. welchii*, *Cl. septicum*, and *Cl. novyi* in the order of frequency. But many aerobes and other anaerobic members of the genus *Clostridium*, such as *Cl. histolyticum*, *Cl. sporogenes*, *Cl. sordelli* and *Cl. fallax*, all present in the soil, are also generally seen in association with these. Recent evidence indicates that *Cl. histolyticum* is also pathogenic to man. Much of the foul odor of gas gangrene is said to be due to *Cl. sporogenes.*

The usual mode of infection is, as in tetanus, through contamination of wounds with infected soil, manure or clothing. As in tetanus again, it is the introduction of spores, and not the vegetative forms, that is responsible for successful infection. But spores or even vegetative forms, washed free of all toxin, cannot attack healthy living tissue. The presence of dead tissue or blood clot at the site of infection is an essential condition for determining infection. These form the suitable anaerobic nidus necessary for the germination of spores and multiplication of the organisms. The anaerobic condition so available is further assisted by the activity of the aerobes. All kinds of wounds with extensive destruction of tissues and contaminated with soil

are very prolific during war and that is why war conditions are particularly favourable for the incidence of gaseous gangrene, as compared to civil conditions.

During the growth of the gas bacilli in the tissues toxins are liberated. They are powerfully histiotoxic. By attacking the surrounding healthy tissue they cause its death. There is no attempt at inflammation but only a simple death which extends very rapidly. This toxic necrosis is the essential process underlying the disease. Large amounts of haemorrhagic fluid accumulate and there is plenty of gas formation with spreading crepitation. The pressure due to these and the results of the consequent impediment to circulation are also subsequent causes for the devitalisation of the muscle. The saccharolytic organisms are the first to be active, but soon the proteolytic ones follow, causing by means of their toxic products digestion and blackening of the dead tissues and further extension of the disease process. The muscles in the affected area are thus rendered soft, friable and black. There is profuse offensive discharge from the wound. Haemorrhagic bullae are formed over the area. A purplish discoloration of the surrounding skin is another marked feature. There is toxaemia from the beginning as the toxins are readily absorbed into the circulation. The organisms, only *Cl. welchii* and *septicum* and not *Cl. novyi*, may invade the blood stream causing septicaemia. The death rate is fairly high, ranging from 20–80 per cent.

Diagnosis. While it is easy to recognise the presence of *Clostridia* in materials obtained from lesions, it is extremely difficult to identify them. The exudate from the infected wound, gangrenous tissue and fluid from bullae are all rich with the presence of these organisms. Films prepared from any of these and stained by the Gram's method will reveal them. By this alone, however, no definite idea of their identity can be gained. That *Cl. welchii* is a short squat organism possessing capsule, may give some indication.

The cultivation of the organisms from the material and their isolation are then proceeded with. The same method as followed in the isolation of the tetanus bacillus is equally applicable in the isolation of the gas gangrene bacilli as well. Fildes' method is not of use in this. For ensuring purity of culture, plating will have to be repeated several times. The organisms may also

be cultivated from the patient's blood. After obtaining pure cultures their identity is to be established by morphology, biochemistry and serology. A presumptive evidence of the presence of *Cl. welchii* in the original material may be obtained by the following method: A suspension of the material in sterile saline is heated at 80° C. for 20 minutes. It is then inoculated into milk and incubated anaerobically. If the organism is present, there will be stormy fermentation of milk. The use of Nagler reaction in the identification of Welch's bacillus has been mentioned above.

Animal experiments are expensive as large number of animals have to be used. The original material, or preferably pure cultures, should be inoculated into groups of protected and control animals. Animals are used in groups of five, one unprotected, three previously protected by inoculating with the antisera of *Cl. welchii*, *Cl. septicum* and *Cl. novyi* separately and the fifth protected against all the three species simultaneously. Evidently, the infecting organisms are those against which the surviving animals had been immunised.

Treatment. The treatment of gas gangrene consists of surgical measures, administration of the specific sera and chemotherapy. Drastic removal of the affected tissues may be necessary and should be boldly performed. Too little and not too much is the risk.

Both polyvalent and monovalent antisera are available. They are prepared in the horse. The mixed serum should be given pending identification of the species and after that the homologous antiserum. It is given intramuscularly or in urgent cases intravenously. Antibacterial sera are also available and a combination of these with antitoxic sera has been reported to give better results. Large doses, three times the prophylactic dose (see below), should be given. That serum therapy is valuable, has not been definitely established. Antitoxic serum against *Cl. welchii* is indicated in puerperal fever due to this organism. This serum is also used by some surgeons as a routine measure before operation in surgical emergencies of the abdomen and in appendicitis.

Penicillin appears to be effective in gas gangrene. Sulphonamide and certain allied compounds have also been reported upon favourably. Transfusion is of great value. But the most important of all is radical surgery.

Prophylaxis. Careful and immediate attention to all fresh wounds is important. All wounds should be carefully cleaned and all damaged tissues removed. The prophylactic administration of antitoxin is useful. The dose is 3,000 international units of *Cl. welchii* antitoxin, 1,500 units of *Cl. septicum* antitoxin and 1,000 units of *Cl. novyi* antitoxin. This standard dose has been recently increased threefold and the prophylactic dose now advocated contains 9,000 units of *Cl. welchii* antitoxin, 4,500 units of *Cl. septicum* antitoxin and 3,000 units of *Cl. novyi* antitoxin.

Chemotherapy is also of some value in the prevention of gas gangrene. Antibiotics and sulphonamides have been found undoubtedly useful in preventing wound infection.

Clostridium Botulinum. This organism was first isolated by Van Ermengem in 1896, while investigating an outbreak of food poisoning in Belgium. Its toxin causes a very serious form of food poisoning called botulism. The ogranism lives mainly in the soil and rarely in the intestine of animals.

Morphology. They are large rods, 0.5–0.8 by 3.0–8.0 microns, with rounded ends, occurring singly, in pairs and in short or rarely long chains. They are motile by means of 4–8 peritrichate flagella, non-capsulated and Gram-positive. The spores are oval and subterminal, causing slight bulging of the mother cell. Some strains produce spores readily, others tardily. Spores are formed best in sugar-free media.

Cultivation. *Cl. botulinum* is a strict anaerobe. The optimum temperature is 35°–37° C. It grows well on ordinary media. Glucose does not improve growth. Both tryptophane and the *sporogenes* factor are necessary for growth.

The organism grows on ordinary media provided strict anaerobic condition is present. On agar surface colonies are large, translucent and irregular with a dense central portion and a thinner reticular periphery. Colonies on deep agar are translucent and fluffy; the central portion may be biconvex, globular or disc-shaped. There is abundant gas production, resulting in the disruption of the medium. In broth the growth is slow but luxuriant in three or four days, with dense turbidity and granular deposit.

Biochemistry. All types ferment glucose and maltose, producing acid and gas. Type A ferments salicin but not Types B and C. Indole is not produced. Milk is slowly digested by

Types A and B and not by Type C. Hydrogen sulphide is produced, but nitrates are not reduced. A haemolysin is produced. The group is not as a whole proteolytic. Gelatin, coagulated serum and egg are generally liquefied by Types A and B; Types C and D digest only gelatin. Meat is digested slowly and blackened by Types A and B; C, D and E are inert.

Resistance. The spores are highly resistant and withstand boiling for several hours but are destroyed by moist heat at 120° C. in five to twenty minutes.

Antigenic Structure. There are five serological types, named from A to E, each producing an exotoxin specific to itself. The antitoxin produced by any of these neutralises only the homologous toxin. The common types responsible for human botulism are Types A and B. Type C was originally isolated from botulism of cattle in Australia and Type D from horses in South Africa. Type E was originally isolated from cases of food poisoning due to consumption of fish; it also appears to cause human botulism. Further, by the use of agglutination and complement-fixation tests these major types can be divided into subtypes which show cross reactions among them.

Toxin Production. *Cl. botulinum* forms a very powerful exotoxin. It is the most powerful of all the exotoxins and is said to be twenty-five times more lethal than the tetanus toxin. It has been computed to be about 10,000 times more poisonous than potassiam cyanide. A dose of 0·00001 ml. injected subcutaneously is enough to kill a mouse. Unlike other soluble toxins, it is markedly resistant to weak acids and on feeding is readily absorbed from the gastro-intestinal system. It is also heat stable, requiring for its destruction a temperature of 90° C. applied for forty minutes. With formalin the toxin can be converted into toxoid. The botulinum toxin is neurotoxic, acting mainly on the parasympathetic system. Although the toxins produced by the various types are serologically distinct, their pharmacological action is substantially identical. Antitoxic sera can be prepared by immunising animals with toxin.

Pathogenicity. The toxins of Types A and B cause botulism in man. The organism itself is not parasitic and need not enter the host to produce the condition. In virtue of their toxins all the types have been found to cause spontaneous disease in animals, such as fowls, ducks, horses and cattle. *Cl. botulinum* causes

limberneck, a paralytic disease of chickens. Certain cases of forage poisoning in cattle and horses have been reported from Australia and U.S.A. Lamziekte, a disease of cattle in South Africa, is botulism.

Experimentally guinea-pigs, mice, rabbits, cats and monkeys are susceptible to toxin administered by mouth or parenterally. Dogs and rats are resistant. The subcutaneous injection of a broth culture into any of these animals causes death in 1–4 days. The symptoms are identical to those of the natural disease.

Botulism (L., botulus, a sausage). Though the organism is widely distributed in the soil, botulism is of very rare occurrence. Outbreaks have been more frequent in Germany and America than in other places. Of the two types A and B responsible for the disease in man, Type A is commoner than Type B. Recently, Type E has also been described from human botulism, particularly from America. The organism grows and multiplies in the food, contaminated directly or indirectly with soil, producing toxin. The disease is caused on consuming such food, uncooked or imperfectly cooked. Although the organism may enter the body through food, there is no invasion of the tissues; it is the preformed toxin that is responsible for the disease which is therefore an intoxication. Bacteria-free toxin will cause the same disease. Preserved food, such as ham, sausage, canned, pickled or smoked meat and canned vegetables, may be responsible. These, when infected, may often evince signs of spoilage; the cans may be blown and there may be a rancid odour on their opening.

It is a very severe type of intoxication and the incubation period is short, from 24–72 hours. The toxin appears to act selectively on the myoneural junctions of the parasympathetic and motor nerves, leading to the production of neuroparalytic symtoms which constitute most of the clinical manifestations of the disease. Whether the nerve cells are affected, is not certain. Vomiting, constipation, paralysis of accommodation and ptosis, pharyngeal paralysis with dysphagia and aphonia and severe prostration are the outstanding symptoms. Death may occur in one to seven days. The mortality rate is high, ranging between 20–85 per cent. or more.

Diagnosis. The bacteriological diagnosis of botulism rests on the demonstration of the toxin in the suspected food. The

food is macerated in saline and filtered through a bacterial filter. The filtrate is injected intraperitoneally into three mice, one previously protected with Type A antitoxin, another with Type B antitoxin and the third unprotected. If A or B and C die, it is positive; the type of the organism is indicated by the surviving animal, A or B. Attempt may also be made to demonstrate the presence of the toxin in the vomit. By appropriate methods the organism may be isolated from the food, vomit and faeces during life and the stomach and intestinal contents after death. The pure culture is then identified by biochemical, serological and virulence tests.

Prophylaxis. Effective sterilisation of the food during preparation for preservation and effective cooking of it before consumption prevent the occurrence of the disease. The food from tins that show evidence of spoilage should not be consumed. To all those who have partaken of the food but not yet developed the disease, 10 ml. of a bivalent antiserum should be administered as a prophylactic measure.

Treatment. Antiserum is not therapeutically very effective. It is administered in 50 ml. doses of the bivalent serum and repeated till the patient recovers. As typing necessarily entails considerable delay, the use of monovalent serum is not practicable. Soap neutralises the toxin and olive oil prevents its absorption. Hence high soap or olive oil enema may be tried. Potassium permanganate destroys the toxin in vitro. Hence solution of this chemical given by mouth may be useful. Alcohol precipitates the toxin and so the administration of it in small doses may have some value.

CHAPTER XXXIII

THE SPIROCHAETES

Spirochaete (coil-hair) is the name applied to a group of elongated, spiral organisms, exhibiting motility without possessing demonstrable flagella. They were first discovered by Ehrenbergh in 1833 in stagnant water. Since then, several types, free-living, saprophytic and parasitic, have been discovered in various situations, such as water, sewage and bodies of insects, animals and man. The spirochaetes exhibit characters peculiar to protozoa on the one hand and bacteria on the other. Chiefly owing to the difficulties of their artificial culture, careful studies have not been possible and so our knowledge about these forms of life remains relatively meagre. Consequently, their biological position has not been finally defined.

Ecology. Free-living, commensal and pathogenic forms have all been met with. The free-living ones are either fresh-water forms or marine forms. The commensals live in the mouth, intestinal canal and genitalia of man and in animals and insects. A few of the pathogenic types are able to lead a free-living existence in water and also a commensal life; the rest are strict parasites.

General Characters. The spirochaetes are slender, elongated, motile, flexible organisms, twisted spirally around the long axis; vary greatly in size and in the number and nature of coils; exhibit no antero-posterior polarity, *i.e.*, they can move forward or backward with equal ease; possess no definite nucleus; multiplication is by transverse division. An important difference from bacteria is the possession of motility without flagella. May be that flagella are present, but too tenuous to be demonstrated by the ordinary methods. The spirochaetes display three types of motility: flexion, cork-screw motion or movement of rotation round the long axis and movement of translation with varying speed. The spirilla differ from the spirochaetes in that they have rigid bodies, preformed coils and locomotion with terminal flagella.

Staining Reactions. The spirochaetes are much less refractile than bacteria; consequently, ordinary hanging-drop method is not useful in their examination. The method of dark ground illumination has to be resorted to for this purpose. It is also more difficult to stain them than bacteria. Hence, special methods have been developed for their demonstration. Negative staining (p. 66) and Giemsa's technique (p. 77) are both useful, but the silver impregnation method gives the most satisfactory results. In this method, metallic silver in very fine particles are deposited on the organisms which in consequence appear black. Levaditi's technique (p. 79) for *Treponema* in tissues and Fontana's (p. 78) for films give the best results. Spirochaetes are Gram-negative.

Cultivation. The cultivation of these micro-organisms is likewise difficult and ordinary media are unsuitable for it. Natural animal proteins are necessary for their growth and so media meant for this purpose should be enriched with blood, serum or ascitic fluid. The spirochaetes are cultivable only on fluid or semi-solid media. A low oxygen tension is essential for growth. This may be ensured by growing them in long narrow tubes containing a fluid medium and a piece of sterile rabbit's kidney and provided with a vaseline seal (Noguchi). The catalase of tissue destroys the inimical hydrogen peroxide that may be formed during growth. Even in this medium growth is rather slow and takes a week or more to appear. *Leptospira* is less exacting in its gas requirement than other pathogenic spirochaetes and so it is less difficult to cultivate. Due to the cultural difficulties, in vivo methods are adopted to maintain in the laboratory strains of spirochaetes; suitable animals, like rabbits, inoculated with them remain as chronic carriers.

Biochemistry. Practically nothing is known about their biochemical properties. The pathogens grow best at 37° C. and are anaerobes; some of them, like *T. pallidum*, are strict anaerobes, while others are less rigid in their gas requirement.

Resistance. These micro-organisms are more vulnerable to the action of destructive agents than the vegetative bacteria. Desiccation is rapidly lethal. The highly pathogenic ones cannot live outside the host tissues for more than a few hours.

Immunity. Like bacteria, the spirochaetes incite the production of antispirochaetal antibodies in the body of the host; agglutinins, spirochaetocidins, spirochaetolysins, complement-fixing

and protective antibodies are all generated. The presence of the last mentioned type of antibody can be proved by protection tests on animals and that of others by appropriate in vivo tests.

A reaction that is peculiar to the spirochaetes and not shown by bacteria is the *Rieckenberg phenomenon* or *adhesion phenomenon.* In this in vitro reaction, the spirochaetes are so altered in their physical properties in the presence of the homologous antiserum that particulate bodies, like platelets and bacteria, stick to them.

The immunity that follows recovery from diseases due to the pathogenic spirochaetes is of low grade and evanescent. But in the course of infection, patients display what is called *infection immunity*, that is, resistance to superinfection with the same organism.

Toxin Production and Pathogenicity. The spirochaetes do not produce any exotoxin, their offensive weapon being presumably the endotoxin or dead and lysed spirochaetal body. The virulence of the pathogenic species is subject to considerable variation. The clinical manifestations they give rise to in man likewise vary widely, some being acute and others subacute or chronic and long standing. The spirochaetes also exhibit some degree of tissue selection; some are mainly blood parasites, while others are chiefly tissue parasites or evince special affinity for the skin or the lymphatic system.

Classification. As our knowledge about these parasites is incomplete and meagre, any classification is necessarily tentative. Several schemes dividing them into distinct genera have been suggested, but all of them are open to objection. Provisionally, we may adopt the following one, classifying all spirochaetes into five genera.

(1) **Spirochaeta.** Large spirochaetes possessing an axial filament round which the body is twisted in a spiral manner, resembling a spiral stair case; no loculation of the body; free-living.

(2) **Cristispira.** Spiral organisms possessing a band-like membrane or crista running throughout the length following the body curve; body is divided into chambers by transverse septa; parasitic in molluscs.

(3) **Saprospira.** Possess neither axial filament nor crista, but body is chambered; free-living or saprophytic.

(4) **Treponema.** Smaller spirochaetes; possess neither axial filament nor crista; having a number of primary coils, closely or loosely wound, secondary coils develop during movement; attenuated or pointed ends in some species; parasitic and many pathogenic to animals and man.

(5) **Leptospira.** Neither axial filament nor crista demonstrable; large number of regular, fine, closely set primary coils; one or both ends frequently hooked; free-living and parasitic, some pathogenic to animals and man.

The spirochaetes pathogenic to man all belong to the two genera *Treponema* and *Leptospira*. The more common and well known ones are listed below:

TABLE XX

The Common Spirochaetes

Treponema pallidum	Syphilis
Treponema pertenue	Yaws
Treptonema recurrentis	Relapsing fever
Treponema vincenti	Incriminated in Vincent's angina, with the fusiform bacillus
Treptonema bronchiales	Incriminated in Bronchitis
Treponema eurygyrata	Incriminated in Dysentery-like condition
Treponema buccalis *Treponema microdentium* *Treponema macrodentium* *Treponema refringens*, present in genitals *Treponema gracile* (*T. callygyrum*)	Non-pathogenic, usually thicker, shorter, with irregular open coils; easily stained.
Treponema cuniculi	Syphilis-like condiion of rabbit, morphologically resembling *T. pallidum*.
Leptospira icterohaemorragioe	Weil's disease
Leptospira hebdomadis	Japanese seven-day fever
Leptospira grippo-typhosa	Swamp fever
Leptospira autumnalis	Harvest fever
Leptospira pyrogenes	Leptospirosis in Sumatra
Leptospira biflexa	Water
Leptospira canicola	Dogs

Treponema Recurrentis. *T. recurrentis*, the causative agent of relapsing fever, was discovered by Obermeier in 1873 during an outbreak in Berlin.

Morphology. They are 10–20 $\mu \times 0{\cdot}2$–$0{\cdot}5\,\mu$ in size with 5 to 10 fairly regular but loose primary coils, each possessing a wave length of 2–3 microns. When seen under dark ground illumination, they exhibit uniform coils and active motility. Romanowsky stains stain them well; Giemsa is the best and imparts a purplish red colour. Carbol fuchsin is also useful. *T. recurrentis* is Gram-negative. Not infrequently the parasite presents granular staining. The stained specimens do not show regularity of curves which tend to open out. Multiplication is by transverse fission. A granular phase has been said to occur in the developmental cycle of the organism, but there is no sufficient proof.

Cultivation. This spirochaete has been successfully cultivated in Smith-Noguchi medium (p. 109). It is very difficult to maintain the culture and some workers even doubt whether the organism has been really cultivated. Growth occurs best under microaerophilic conditions. The optimum temperature for growth is 30° C.

Classification. The relapsing fever spirochaetes have been placed in a separate genus *Borrelia* by Bergey. In view of their close similarity to *T. pallidum*, the creation of a new genus is open to question. Many authorities include them in the genus *Treponema*.

Slight differences in the immunology and virulence to laboratory animals are displayed by the various strains occurring in different regions. On this ground and on that of certain minor clinical dissimilarities, distinct specific names have been given to them, such as *T. duttoni* in West and Central Africa, *T. berbera* in North Africa, *T. novyi* in North America, *T. venezuelensis* in South America, *T. obermeieri* in Europe, *T. carteri* in India and several others. Such minor variations do not really constitute sufficient criteria for the creation of new species and these different strains are better considered as no more than different races of the same species, *T. recurrentis*.

Transmission. *T. recurrentis* is essentially a blood parasite and its transmission is by blood-sucking insects. On this ground, relapsing fever has been divided into two epidemiological groups, the louse relapsing fever and the tick relapsing fever.

The louse responsible for transmission is the body louse, *Pediculus corporis*. It is the vector in Europe, India and North

America. Infection of man occurs not through direct bite but by the contamination of the bite-wound with infected excreta of the insect or by the rubbing in into the wound the crushed body of the louse during scratching caused by irritation; the insect's body fluids contain the spirochaetes. In the louse, transmission takes place through blood sucking and possibly also hereditarily. After the infective feed, spirochaetes can be demonstrated in the stomach for twenty-four hours, after which they disappear. About six days after, they begin to reappear in the body cavity and thereafter are found distributed in all parts of the body. A louse, once infected, remains so for the remainder of its life and hereditary transmission through eggs may occur.

The tick is the transmitting agent of relapsing fever in Tropical Africa and Central and South America. Different species of the soft tick, *Ornithodorus*, are responsible in different localities. Man is infected by infection of the bite puncture with the infectious excretion of the coxal gland or with infected excreta. Of these, the former is probably more important in transmission. As in louse infection, the parasites disappear from the gut of the tick subsequent to feeding on infected blood and after an interval reappear in the tissues. An infected tick remains infected throughout life, and the occurrence of hereditary transmission of infection in these insects has been established.

Pathogenesis. *T. recurrentis* causes relapsing fever in man. The organism is readily transferable to monkeys, in which a disease clinically identical with relapsing fever in man can be produced by injection of the organism. Mice and rats are susceptible but less readily than monkeys; guinea-pigs and rabbits are refractory. The parasite is present in their blood during the febrile period. The West African strains (*T. duttoni*) are said to be more virulent to monkeys than others.

An identical spirochaete, *T. gallinarum* (or *anserinum*), occurs as a natural infection in fowls, geese and ducks. *T. theileri* is associated with a natural disease of cattle in South Africa.

Relapsing fever is an acute infectious disease due to *T. recurrentis* and is prevalent in all continents. In India, the Nilgiris, around Madras City, the United Provinces and North West India are endemic centres. The disease is associated with overcrowding, famine conditions, dirt and insanitation. Sometimes it breaks out in epidemics.

The incubation period varies from 5–10 days. The clinical picture is the same in all forms of relapsing fever. A sudden onset with chills, high fever, severe frontal headache and muscular and joint pains constitute the usual symptom complex. There is moderate enlargement and tenderness of the spleen. The fever lasts for a few days, 3 to 5, and falls by crisis. It is followed by an apyrexial period of about two to fourteen days. The symptoms start again and the cycle continues. As many as ten relapses may occur in the course of an infection. The tick relapsing fever of Central and West Africa is said to be more serious than the louse relapsing fever. During the pyrexial period of the illness, the spirochaetes are present in varying numbers in the peripheral blood; during the quiescent phase they disappear from it, but they are present in the internal organs, like spleen. The occurrence of *T. recurrentis* in the cerebrospinal fluid has been reported. The mortality rate varies from 5–30 per cent.

Immunity. Lytic and agglutinating antibodies appear in the blood in a few days after the onset of the illness. The disappearance of parasites from the peripheral blood, almost synchronising with the fall of temperature or a little earlier, is probably due to the destructive action of the antibodies on spirochaetes. A few may, nevertheless, lurk in the brain or some other invulnerable islets during the apyrexial period and, with a fall in the titre of the antibodies, may reinvade the blood, causing relapse. This is repeated till the active immunity reaches a sufficiently sustained high potency to eradicate the infection. The relapses are progressively milder; finally most cases recover. Another explanation of relapse is the development of antigenically variant strains in the tissues, which on reinfection of blood produce relapses. The relapse-strain is said to be antigenically distinct from the primary infection-strain. As in syphilis and tuberculosis, there is infection immunity also in relapsing fever, and superinfection during the course of the illness fails to occur. Sera from patients suffering from relapsing fever may give a positive Wassermann reaction. The Rieckenberg reaction is positive (p. 627). The immunity left after an attack of relapsing fever is of low grade and subsequent attacks may occur.

Diagnosis. The organisms are seldom numerous in the peripheral blood, but they do occur. During the febrile period the

examination of blood under dark field illumination may yield positive results; so also the examination of thin and thick films stained by the Leishman or Giemsa technique or with dilute carbol fuchsin. It is worth bearing in mind that these spirochaetes in stained smears do not maintain their regular form; they may be uncoiled, curved or in small bunches. If these methods fail, blood may be inoculated into mice; after a few days, spirochaetes appear in large numbers in their peripheral blood. Smears should be examined daily for at least two weeks if the results are negative. It should be remembered that these rodents may have natural infection with similar spirochaetes.

Serological reactions are not of much diagnostic help. Recently, positive complement-fixation and agglutination reactions have been reported with a specially prepared spirochaetal antigen and the patient's serum.

Prophylaxis. The improvement of the general conditions that favour the spread of infection, campaign against lice and ticks and the avoidance of their bites are the main lines of prophylaxis.

Treatment. Arsenical compounds are curative. It is stated that the spirochaetes rapidly develop arsenic-resistance. Hence, treatment should be with large doses at short intervals.

Treponema Vincenti. Vincent's spirochaete is a delicate actively motile organism with a measurement of 5–10 microns long and 3–8 irregular spirals. It is Gram-negative, but carbol fuchsin is the best stain to demonstrate it. It is anaerobic and can be cultivated at 37° C. on serum agar or serum broth. Smears from Vincent's angina, ulcerative stomatitis, tropical ulcer (Naga sore) and allied conditions show large numbers of these spirochaetes, practically always mixed with the fusiform bacillus. Their aetiological relationship to these conditions has not been determined. There is no proof that Vincent's spirochaetes are actively pathogenic and are primarily responsible for any of these conditions. It has been stated that these spirochaetes are developed from the fusiform bacilli and that the two are different phases of a single micro-organism, but this is based on very slender evidence. They appear to be distinct organisms living in symbiotic relationship with one another. Arsenicals are of value in the treatment of Vincent's angina. Local penicillin therapy has been reported upon favourably.

Treponema Bronchialis. A condition called bronchial spirochaetosis has been described by Castellani, but it is doubtful whether a separate clinical entity like this really exists. The spirochaetes that occur in this condition are not always of a uniform type nor do they show any morphological or other characters distinct from the spirochaetes occurring in the mouth. Hence, the creation of a separate species does not appear to be valid.

The relationship of *T. eurygyrata* to the dysentery-like condition ascribed to it and its general characters are both uncertain as in the case of *T. bronchialis*. It is said to be a dwarf spirochaete possessing only two coils.

Non-Pathogenic Treponemata. *Treponema refringens* is a large, motile, spiral organism, 10–30 microns long, with relatively few, irregular, sweeping coils. It is easily stained and is Gram-negative. It is found on normal mucous surfaces and also in necrotic and ulcerative lesions anywhere on the body. The organism may occur in syphilitic sores and has then to be differentiated from *T. pallidum*.

Treponema gracile (or *callygyrum*), occurring in secretions of the genitals, bears a close morphological resemblance to *T. pallidum*. It is slightly stouter than the latter, possesses narrower coils and stains more readily. It shows a glistening appearance under dark ground illumination, whereas *T. pallidum* is dead white. Several species of spirochaetes have been described as normal inhabitants of the mouth. Spirochaetes displaying close resemblance to *T. pallidum* are present in considerable numbers in carious teeth, pyrrhoeal secretions and in chronic ulcerative conditions of the mouth. One of them, *Treponema microdentium*, is hardly distinguishable from *T. pallidum* save for its shorter length. *Treponema macrodentium* is another, but it is thicker than *T. pallidum* and its coils are less regular. These organisms are more readily stained by the ordinary methods than the pathogenic spirochaetes.

Treponema Pallidum. *T. pallidum*, the causative organism of syphilis, was discovered by Schaudinn and Hoffmann (1905) in materials from chancres of syphilitic patients. The discoverers had called the new organism *Spirochaeta pallida*, but it was held untenable as the name *Spirochaeta* had been previously applied to a different organism. Vuillemin subsequently suggested the

name *Spironema pallidum* to the new organism. It was then found that the generic name *Spironema* had objection too on the ground of its prior application to a flagellate organism. Finally, as Schaudinn considered the newly discovered spirochaete a protozoon, he renamed it *Treponema pallidum* (p. 353).

Prior to the discovery of *T. pallidum*, several attempts had been made to reproduce syphilis in experimental animals, with some success in the rabbit by Haensell (1881) and in the higher apes by Metchnikoff and Roux (1903). Within a decade of the discovery of the orrganism, several important advances were made in syphilology. Wassermann and collaborators introduced in 1906 the invaluable serum test, now known as the Wassermann test. The next notable contribution was made by Ehrlich in 1910 by the discovery of salvarsan. In 1911 Noguchi succeeded in cultivating the organism in pure culture in artificial media and two years later he demonstrated its presence in the brain tissue of cases of general paralysis.

T. pallidum is a strict parasite and lives in the lesions it causes. In discharges and tissues removed from the body, the organism may be seen alive for a brief interval.

Morphology. It is a delicate organism, about 6–14 microns long by 0·25–0·3 micron thick, with finely tapering pointed ends and some 10 regular closely set coils, each having a depth and amplitude of about 1·0–1·2 microns. The central coils are slightly more compact and broader than the extreme ones. As the organism has very low refractile power, dark field illumination is necessary to demonstrate it in the unstained state. It has a dull silver-white appearance quite distinct from the brilliant-white glistening appearance of *T. refringens*. Its movement of translation is very sluggish and the organism tends to remain in the same spot in the field, but rotatory and flexion movements are conspicuous features, particularly the latter. Reproduction is by binary transverse division.

No flagella are demonstrable by ordinary methods, but recently claims demonstrating a terminal flagellum at each pole have been put forward. The presence of flagellar structures has also been noted under the electron microscope. The existence of a granular filterable phase has likwise been suggested. But the evidence in support of these claims is inadequate.

T. pallidum is refractory to the ordinary methods of staining. The indirect or the relief method is useful; in this the background is stained, leaving the organism unstained. A drop of fluid containing the spirochaete is mixed with a drop of Indian ink or Congo red, spread on the slide, dried and examined (p. 66). Giemsa's stain is employed in one of two ways: staining with a 1 : 10 dilution of it for twenty-four hours or with a 1 : 2 dilution for one hour. The organism is tinged light pink. Fontana's silver impregnation method gives very satisfactory results. Levaditi's method is employed for staining the spirochaetes in tissues (p. 79). Both stain the organism black.

Cultivation. It is very difficult to grow the organism artificially. In fact, doubt has been cast on Noguchi's claims of cultivation. Rigid anaerobic conditions are indispensable for its growth. Enrichment of the medium with serum or ascitic fluid and the addition of a piece of sterile fresh tissue, usually rabbit kidney or testicle, are necessary. The optimum temperature is 37° C. Growth is slow. Due to the difficulties of cultivation, the usual procedure to maintain strains is by inoculating infective materials from cases into the testes of rabbits. The organism is readily obtained in a pure state from a popliteal gland excised from the infected rabbit. Strains may also be maintained in the mouse.

Resistance. *T. pallidum* is readily destroyed by the common antiseptic agents. Heat at 41·5° C. kills it in one hour: on this is founded the modern pyrotherapy in syphilis. Cold is resisted longer; the organism remains viable in citrated blood for three days at 5° C. Drying is particularly lethal. In discharges removed from the body and left to dry, the organism perishes quickly in a few hours, but in tissues so removed it lives longer.

Immunity. Man does not possess any natural resistance against syphilis and all uninfected individuals exposed to infection are usually infected. Escape from infection need not necessarily prove the presence of immunity against syphilis, for such escape is possible if no local trauma to let in infection is caused during exposure. An unsuspected latent infection, congenital or acquired, may also prevent superinfection. A certain degree of community resistance is undoubtedly developed against syphilis by long contact. When first introduced into a community, the disease

runs a malignant course similar to that of any acute infectious disease, but in course of time, as the result of long contact, it assumes a subacute or chronic type as we know it today. There is also the possibility, contentious as it may be, that the decrease in the severity of the disease might also be an expression of a decrease in virulence suffered by the parasite during the protracted process of a host-parasite adjustment.

The existence of racial differences in natural immunity against syphilis has been suggested, but it is based on slender evidence. Women are said to develop acquired immunity in a higher degree than men; this also has yet to be substantiated by convincing proof.

In syphilis some sort of immunity undoubtedly develops. As in tuberculosis, it is of the infection immunity type not dependent on the presence of antibodies in the blood. After the disease has established itself, superinfection, as evidenced by the production of a primary lesion, is not ordinarily possible and such resistance continues so long as the individual harbours the infection. During the incubation period of syphilis, *i.e.* prior to the development of primary chancre, when immunity has had no time to develop in sufficient force, superinfection is easy. Clinical experience testifies to this; multiple chancres may form at this stage. Fresh exposure during this period likewise results in superinfection with the production of a second chancer. The same result can be obtained at this stage by experimental inoculation. By the time primary chancre has well developed, sufficient immunity also will have been produced. After this, so far as can be judged from the production of clinical symptoms, there is increasing resistance to further infection, whether natural or artificially induced. It is a common clinical observation that the development of a second chancre as the result of second infection is extremely rare in the course of the disease after the primary chancre has developed. But it is to be noted that though the characteristic local lesion fails to develop on reinfection, it cannot be denied that the spirochaetes may be causing symptomless infection. In other words, any absence of infection may be only apparent and not real. Even so, the course of infection is undoubtedly modified. Again, there is evidence that syphilitics can be completely cured with efficient treatment in the early stages. Such recovered individuals are again susceptible to fresh

attack, indicating that with complete recovery acquired immunity disappears. The presence of a latent stage in the clinical course may be a further proof of the development of immunity. These observations are indicative that in syphilis the tissues of the host develop and maintain a certain degree of immunity so long as the infection persists. This acquired immunity never attains a high grade. Once active symptoms have developed, syphilis does not show any tendency to spontaneous cure. That shows that the immunity response never rises to a degree adequate to eliminate infection altogether from the patient. It is true that in some people the infection is latent throughout, but in them serum reactions may be positive and the condition is always a potential danger.

In syphilis ordinary antibodies, like agglutinins, lytic factors and immune opsonins, are not produced: at any rate their presence cannot be demonstrated by employing the avirulent culture strains of *T. pallidum* (p. 644). The occurrence and nature of the syphilitic antibody responsible for the serum reactions are discussed below. As already mentioned, complete recovery, when achieved, leaves the patient as vulnerable as before infection.

Much of our knowledge about immunity in syphilis, its nature and mechanism, its duration, its influence on cure or *vice versa*, is mainly based on the results of animal experiments. Rabbits, when inoculated with *T. pallidum*, develop chancre after a certain interval. After this event, there is increasing difficulty in reproducing chancre by reinoculation with *T. pallidum* and a stage is reached when it entirely fails, showing thereby that, as the result of long-standing infection, a low grade immunity is established in the rabbit. Rabbits, reinoculated prior to the development of the primary chancre, invariably develop the characteristic local lesion. The same results are obtained when rabbits adequately treated with arsenic compounds in the early stages are reinjected subsequently with the same strain of the organism. Treatment in the late stages of inoculated syphilis in rabbits does not seem to eradicate the infection, nor do such cases recover spontaneously. All these points are still largely unsettled, and it is not certain whether the results of experimental studies on rabbits can be applied without qualification to human syphilis.

Toxin Production. Our knowledge about the exact mode of production of syphilis is still meagre. Nothing is known

regarding the toxic factors of *T. pallidum*, whether it produces any exotoxin or what kind of endotoxin is contained in the organism. Similarly, nothing is known about its antigenic structure and whether it undergoes or not any variation like bacteria.

Pathogenesis. *T. pallidum* is a highly invasive organism. It causes syphilis in man. No other animal is naturally infected. But a somewhat similar condition is found to occur naturally in rabbits due to *T. cuniculi* which is morphologically indistinguishable from *T. pallidum.*

Experimentally the infection can be reproduced in anthropoid apes, rabbits and mice. In monkeys, the infection is introduced through the scarified skin of the eyebrows or of the genitalia; infected tissue may likewise be implanted under the skin. By these procedures, lesions typical of primary and secondary lesions in man are produced. Rabbits, however, are more convenient; they are inoculated into the anterior chamber of the eye or into the testis. The former causes keratitis and iritis and the latter orchitis after an incubation period of two or three weeks, followed by involvement of the inguinal glands and general dissemination of infection. Injection into the skin of the scrotum gives rise to a sore resembling chancre. The occurrence of a natural infection of rabbits with *T. cuniculi*, referred to above, should be kept in mind lest false results should be recorded. The inoculated mice do not show any lesion, but they take the infection which remains inapparent indefinitely.

Syphilis must have existed from ancient times. But there is no authentic record of its clinical recognition or prevalence prior to the closing years of the fifteenth century when the sailors of Columbus returning from the New World were supposed to have introduced the disease into Europe. Whether the disease was really exotic to Europe before this period, is a disputed point. Whatever that be, Europe witnessed at this time widespread outbreaks of the disease. Any authentic history of the disease dates from this period. The disease derived its name from "Syphilus", the hero of a poem written by Fracastorius in 1530, who was supposed to have acquired the disease in a fell form because of his improvident ways.

Syphilis is widely prevalent throughout the World. Its exact incidence rate is not known; an infection rate of about 8–10 per cent. of the general population has been reported in

some of the Western countries. Between 1 and 2 per cent. of the children in the U.S.A. are computed to have congenital syphilis. Syphilis does not make any racial discrimination; the sexes are also equally susceptible. Climate, seasons and similar factors play no role in its incidence. Opportunity for contact, economic and social status, mode of living and personal habits are some of the factors that influence its spread and natural course. When the infection is newly introduced into a hitherto uninfected community, it behaves as any other acute infectious disease, running a virulent course and causing high mortality. This is stated to have happened in Europe on its first introduction in the closing decade of the fifteenth century. After long contact, the conditions of resistance in the host species appear to have undergone changes, bringing about clinical modifications in the natural history of the disease which is now much less acute but more insidious.

It has been stated that there are two types of *T. pallidum*, one with special affinity for epithelial structures, the dermotropic type, and the other possessing special affinity for the nerve tissue, the neurotropic type. More experimental and clinical data are necessary before this can be accepted.

The effects of pregnancy on syphilis are twofold; it alters the clinical nature of the disease in the pregnant woman and it causes spread of infection to the progeny. The natural physiological changes of pregnancy are held by some to be operating in favour of the individual against the disease. The serology may be masked during pregnancy. Others regard that pregnancy is not always protective and that the stress and strain associated with it may operate in the opposite direction. The spread of infection to the foetus can take place only on one condition and that is the presence of treponemata in the pregnant woman's blood. A healthy, intact placenta may probably offer some barrier, but the parasite may reach the foetal circulation even in the absence of detectable lesions in that organ. The transfer of infection through spermatazoa does not seem to occur and consequently transmission by father does not take place except indirectly by infecting mother. In the late stages of the disease, in latent cases and where the woman acquires the infection very close to the time of delivery, the offspring may escape infection. In others, where there are circulating organisms in the mother,

syphilis is transmitted to the foetus. Transmission may take place any time after the sixteenth week of pregnancy.

Thus, in syphilis there are two important methods of transmission of infection—direct contact through sexual union and placental transmission from mother. Extragenital direct contact, such as happens by kissing, wet nursing, circumcision or during examination by the physician, is a much rarer mode and transmission through blood transfusion is far rarer still. Direct implantation of the organism into minute breaches in the epithelial barriers of the skin or mucous membrane of the urogenital tract is necessary for successful infection. On this account and also because *T. pallidum* is a very delicate organism not capable of living outside the tissues for more than a very brief period, transmission through fomites is extremely rare. However, the possibility that the organism is capable of penetrating the intact mucous membrane cannot be precluded. About 90 per cent. of cases of acquired syphilis have been ascribed to sexual contact and the rest to extragenital methods.

Syphilis is a chronic infectious disease of long duration. It is one of the infective granulomas. It is conveniently classified into acquired syphilis and congenital syphilis. The incubation period of acquired syphilis is about three weeks with outside limits of 10 and 90 days. The course of the acquired disease is conventionally divided into three stages—primary, secondary and tertiary; sometimes a quaternary stage also is described. Convenience for description is the only justification for the continuance of this arbitrary division. *T. pallidum* is very invasive and infection is a continuous progressive process from the beginning. And it is as much difficult to determine the exact time of general dissemination during the evolution of the illness as it is to decide the time of transition from the secondary stage to the tertiary. In fact, there is evidence indicative of a much earlier generalisation than was known before. Now the disease is divided into early syphilis and late syphilis including latent syphilis.

At the end of the incubation period, the clinical course is ushered in by the appearance of the primary lesion, the Hunterian chancre, at the site of infection. Subsequently, it breaks out into an indolent, indurated, painless, exuding sore, both sore and discharge teeming with the treponemata. The regional glands

enlarge and show the presence of the parasite. Ordinarily, the sore heals in four to six weeks; as it tends to heal, the spirochaetes in it become less and less numerous.

Soon after infection, general diffusion of the organism into the body occurs. Spread takes place by way of the lymphatics and later through the blood. Septicaemia soon supervenes. Syphilis is protean in its manifestations. No tissue in the body is exempt from its attack. However, certain tissues and organs, such as the skin, mucous membranes, central nervous system and cardiovascular system, are particularly picked up by the parasite for its ravages. Sore throat, cutaneous rash, bone and joint pains, generalised enlargement of the lymph glands and often fever constitute the main symptomatology during the stage of established generalisation or the clinical secondary stage. *T. pallidum* is present in all viscera and lesions but particularly in great numbers in condylomata, lymph glands, mucous patches and cutaneous rashes; the parasite has been demonstrated in semen and the cerebrospinal fluid. Obviously therefore, this is the most infective period.

Probably with the development of immunity, the early stage of generalised infection passes on insensibly into the late stage, in which infection settles itself down in certain localised areas. The clinical manifestations of the disease at this stage are very varied and no characteristic picture can be drawn. This stage may last for years. A proportion of cases may remain quiescent. The essential pathological changes are chronic endarteritis, perivascular inflammation and fibrosis, leading to destruction of tissues in the affected organs and other situations. Gummatous formation may likewise occur in various parts of the body including the viscera. The cardiovascular and nervous systems may be the seats of such destructive changes and their involvement declares itself more commonly during this stage. In neurosyphilis any or all the constituent structures of the nervous system, the vascular, supporting or nervous tissue proper, or any part of it, the central nervous system or the peripheral nerves, may be affected. Again, the affection may be localised or widespread. The meningovascular or the interstitial form is earlier to appear and the parenchymatous form, general paralysis and tabes dorsalis mainly, later. The number of organisms present in late syphilis is few and it is increasingly difficult to demon-

strate them as the disease progresses. By careful search they can be seen in the periphery of the gumma and in the vascular lesions: treponemata have been demonstrated in the cerebral cortex. in general paralysis, in the spinal nerve roots and spinal cord in tabes dorsalis and in the aortic wall in aortitis.

The central nervous system may be invaded very early in the course of the infection, but symptoms may not be apparent at this stage. The exact time of its involvement is difficult to determine. Neurosyphilis, however, is generally a manifestation of the late stage of the infection. With involvement of the central nervous system the cerebrospinal fluid may show pleocytosis, increased protein (globulin) content and positive serology.

A minority of cases do not exhibit any clinical evidence of infection from the commencement; they constitute latent syphilis, the only indication of infection being positive serology, accidentally detected. In some of these, a break down of the defence mechanism may activate the infection at a later date and lead to clinical syphilis, while in the rest the latency continues throughout life.

Congenital syphilis is from the beginning a systemic infection and treponemata are widely disseminated in the body. The foetus may die in utero, the infant may be born with frank clinical manifestations or it may be apparently normal. Large number of spirochaetes are present in the lesions and in the internal organs: they are scanty in the latent type which has a clinical, bacteriological and immunological correspondence with the latent syphilis in adults. Affection of the nervous system is much less frequent in congenital than in acquired syphilis. The serological tests are uniformly positive in all cases of congenital syphilis, except in those of the latent type, in which the percentage of positives varies. Due to a breakdown of resistance, these inapparent cases may later develop symptoms: but few of them may remain latent even throughout life. Adult women with such latent infection only rarely transmit it to the foetus, but third generation syphilis does occur, although rare.

Diagnosis. The early diagnosis of syphilis is extremely important. The demonstration of *T. pallidum* in the exudates and tissues constitutes the surest proof, but this is not always possible and serological tests are then of the greatest help.

It is relatively easy to demonstrate *T. pallidum* in the serous exudate from lesions. It is done most conveniently by the

dark ground illumination technique or, failing this, by staining (p. 626).

The differentiation of *T. pallidum* should be carefully made. The silvery white appearance, regularity of spirals and pointed ends, sluggish motility the organism tending to stick to one spot in the field and the flexion movements are all important details distinguishing it from *T. gracile* and *T. refringens* with their thicker size, glistening appearance and more active motility.

When collecting serous exudate for examination, care should be taken to exclude, as far as possible, all contaminating organisms usually found on the surface. This can be accomplished by taking serum from beneath the surface of the sore. Blood-stained fluid is not suitable, for the presence of red cells will tend to obscure the spirochaetes; what is wanted is the clear serous exudate. The organism will be scanty or absent if any local antiseptics had been applied before. For these reasons in all cases of open sore, after a thorough cleaning, a sterile saline dressing is applied for twenty-four hours prior to taking material. After this, the sore is cleaned with warm sterile saline, dried and scraped inside the margin gently by a blunt scalpel or the edge of a slide. Blood-stained fluid will exude; it is wiped off once or twice and in a few seconds clear serum will follow. A drop of the exudate is removed to a thin slide, a cover-slip is applied and rimmed with vaseline and the preparation is then examined. If immediate examination is not possible or if the specimen has to be despatched to a laboratory, the serous exudate is aspirated into one or two capillary tubes, sealed on a flame and despatched without delay. The specimen should be examined without loss of time, for *T. pallidum* removed from tissues does not remain viable for a long time. Similarly, aspirated fluid from buboes or serum obtained from the secondary skin lesions, such as condylomata, mucous patches and others, may be examined.

Serum Reactions. Both complement-fixation and flocculation tests find extensive application in the diagnosis of syphilis. The luetin reaction or the allergic intradermal test is seldom done because of the irregularity in its results.

Wassermann Test. The Wassermann test is only a special application of the principle of complement-fixation reaction (p. 290) in the diagnosis of syphilis; the test is named after Wassermann who with his colleagues first applied the complement-

fixation reaction for this purpose. The reaction depends upon the presence of a lipoidophilic substance in the syphilitic serum. And in doing the test we are actually attempting to discover the presense or absense of this substance in the blood or cerebrospinal fluid of a suspected case. It is one of the most reliable and valuable diagnostic tests.

Wassermann and his collaborators originally employed as antigen a watery suspension of syphilitic foetal liver containing *T. pallidum*. With this they obtained positive reactions with syphilitic sera and negative reactions with normal sera. Subsequently, it was found that an extract of non-syphilitic liver fixed complement equally well in the presence of syphilitic serum; later still an alcoholic extract of muscle tissue was found a better substitute. The exact nature of the tissue extract is not known: it consists of lipoids (phosphatides) soluble in alcohol and the reacting substance is most probably the lecithin fraction. Of all the lipoids investigated from various tissues, those from the heart muscle were found to possess the best antigenic quality. At the present time an alcoholic extract of bovine heart is the one that is commonly employed; sheep's heart and human heart are also used. Though cholesterol does not possess any antigenic value, its addition to the heart extract renders the latter more sensitive; a one per cent. alcoholic solution of it is employed in the proportion of three of heart extract to two of cholesterol solution.

There is considerable practical difficulty in employing a treponema antigen for the Wassermann test. However, reactions have been conducted employing both saline suspensions and alcoholic extracts of the culture strains as antigens, but these were found to be less sensitive than the tissue lipoids. Recently, the Reiter strain of *T. pallidum* has been reported to yield strong agglutination and complement-fixation reactions with syphilitic sera. How far it is superior to the tissue extract and whether it can be of practical value in view of the present unsatisfactory methods of cultivation, are questions yet to be decided.

The Wassermann Antibody. What exactly is the nature and mechanism of production of the substance present in the serum and cerebrospinal fluid in syphilis, that is responsible for the positive complement-fixation and flocculation reactions, is unknown. That it is a regular antibody produced against, and

inimical to, *T. pallidum* is doubted. Hence the name *Wassermann antibody* or *reagin* is given to it. Though not conclusively proved, it is generally agreed that the antibodies concerned with the complement-fixation and flocculation reactions are the same. As in the case of other antibodies, the Wassermann antibody is probably associated with globulins. It is not destroyed at 55° C. It is a lipoidophilic substance that can interact in vitro not only with the lipoids of treponemata, but also with tissue lipoids which are antigenically similar. Hence, it has been suggested to be a heterophile antibody (p. 235). There are several theories regarding the mechanism of its production, but none satisfactory. One is that it is the product of tissue breakdown resulting from the activity of the treponema or it is a true antibody indirectly produced as the result of stimulation by such disintegration products. Some regard it as a true antibody provoked by a specific antigen residing in *T. pallidum.* The presence of the Wassermann antibody in the blood is not regarded as an expression of immunity; rather it is considered as an indication of the presence of *T. pallidum* in the body, its concentration in the blood, as evidenced by the degree of positivity of the serum reactions, varying in proportion to the number of treponemata present in the tissues.

From what has been said above, it will be obvious that the Wassermann reaction is non-specific in the biological sense, as the antigen, not being derived from the causative organism *T. pallidum*, is not specific. But that does not in any way detract from the reliability and consequently the value of the test. The explanation of the reaction is not clear. It is possible that the tissue lipoids are antigenically similar to some lipoidal hapten contained in *T. pallidum* and that chemical specificity, therefore, really underlies the reaction. Though many serological tests have been devised for the diagnosis of syphilis, the Wassermann test remains still the most reliable.

The Wassermann technique is a complicated one. There are several modifications of it, but the underlying principle is the same in all. The test is done in two ways. Many laboratories employ constant amounts of antigen and of patient's serum with varying amounts of complement. In others the amounts of antigen and of complement are kept constant, while the amount of serum is changed. In both the general procedure is the same.

Accurate standardisation of the reagents is very important. A standard volume is employed for all reagents, and the test is performed in small standard test tubes. The antigen and the inactivated serum are mixed in proper proportion by means of fine graduated pipettes. To this mixture the complement is added and the tubes shaken. The mixtures are then incubated at 37° C. for one hour, shaking them once or twice in the interval. At the end of this period, the haemolytic, or the indicator, system (p. 291) is added and the tubes are shaken and put back in the incubator for another hour, again shaking them once or twice during the period. The racks are then removed from the incubator and left on the bench for a couple of hours or in the refrigerator overnight, after which the results are read. In a positive case the antigen-antibody complex would have fixed the complement, leaving no free complement in the system. The sensitised cells are, therefore, left intact, *i.e.*, there is no haemolysis. The cells form a deposit at the bottom and the supernatant fluid is clear and not discoloured. The absence of haemolysis is thus indicative of a positive reaction. In a negative case, on the other hand, owing to the absence of the immune body, no antigen-antibody complex is formed and so there is no fixation of complement. The complement is thus free to act upon the sensitised cells which are in consequence lysed, causing a uniform discoloration of the fluid with no sedimentation of cells. The presence of haemolysis, therefore, signifies a negative reaction. For details of the test refer to books on practical bacteriology.

The serum reactions are negative in the first one or two weeks of syphilis. After this, positive reactions begin to appear, but negative reactions are also frequent. As the disease progresses, the reaction becomes increasingly positive. Some authorities give 70 to 80 per cent. positives for all cases of untreated primary syphilis. The finding of *T. pallidum*, however, should be the most important criterion of diagnosis in this stage. During the clinical secondary stage, practically all untreated cases show positive reactions. Despite this, correlation of the clinical and laboratory findings is very important for arriving at a correct diagnosis. With the advent of the late stage, the frequency of positive reactions diminishes, varying with the activity of the disease. Where active lesions are present, the incidence of positives is very high (75 per cent.), but when the disease is chronic and latent, it is low

(25–50 per cent.). In meningovascular syphilis the incidence of positives is about 60 per cent., slightly higher in tabes dorsalis and 100 per cent. in general paresis. From the above, it is evident that a negative reaction in the primary stage of the illness and in tertiary and latent cases does not exclude syphilis. But a repeated negative test in suspected secondary syphilis practically always excludes the disease.

Congenital syphilis with manifest lesions is almost always positive. In the absence of these and in latent congenital infection, the percentage of positives is low, not more than sixty. It is preferable to do the test on the infant two to three weeks after birth, so that the possibility of a false positive from the presence of maternal antibodies, transferred through placenta, may be eliminated. Though the mother of a congenitally syphilitic child need not always give a positive serum reaction, testing her blood is often helpful and necessary.

Both acquired and congenital syphilis may yield positive reactions with the cerebrospinal fluid. But the time when the central nervous system is invaded by the parasite causing a positive reaction in the cerebrospinal fluid cannot be definitely determined. It is stated to occur very early in the course of the illness. In cerebrospinal syphilis (neuro-syphilis) a high percentage of positives is got in the spinal fluid, and about 70 per cent. in tabes dorsalis; but in these the serum reaction does not run parallel and is often negative. In general paresis both the spinal fluid and blood show 100 per cent. positives. The indication, therefore, is to examine both the blood and spinal fluid in all cases of suspected central nervous system implication.

A positive Wassermann reaction is obtained in yaws and trypanosomiasis and probably in rat-bite fever, relapsing fever and tropical eosinophilia. Positive reactions have been likewise reported in leprosy, malaria and infectious mononucleosis. But the possibility of associated syphilis giving positives should always be borne in mind.

False positive reactions, aside from certain diseases mentioned above, are usually due to technical errors. Apart from this, bacterial contamination of serum may give rise to false positive reactions; so also badly haemolysed specimens. Even in the best of circumstances, however, a small proportion of false positives, less than 1 per cent., is obtained with the blood of

apparently normal individuals, for which no explanation can be found.

False negative reactions are mainly due to two reasons—one technical and the other low concentration of reagin in the serum. Any test that gives a positive reaction rate of 80 per cent. or more is deemed to be a satisfactory one. Apart from the method, technical errors are often the source of false negative reactions. A low concentration of reagin may occur at any stage of the disease but is more common in the early primary and late and latent stages. A low titre in the chronic stage indicates inadequate stimulation consequent on a localisation of the infection. The titre of the reagin is said to fluctuate in latent cases, accounting for the irregular results rarely seen in the same individual at different times.

As a result of treatment the majority of individuals become serologically negative in due course. The time taken for the disappearance of a positive reaction is contingent on several factors, such as the individual, the stage of the disease, the efficiency and continuity of treatment and the sensitivity of the test employed. It varies considerably. In general, the majority of patients in the primary and early secondary stages become negative after one or two courses of treatment. A few may become negative even after one or two injections, some remain positive for months and may then become negative without further treatment, while a certain proportion, about 10 per cent. remains positive indefinitely—the so-called Wassermann-fast individuals. The explanation of this variation is by no means clear.

Precipitation or Flocculation Tests. Michaelis discovered (1907) that precipitation occurred in syphilitic serum on the addition of tissue extracts. Since then, numerous tests based on this phenomenon have been devised for the serum diagnosis of syphilis: sachs-Georgi, Sigma, Kahn, Meinicke, Vernes, Kline and others. In all likelihood, the flocculation tests are dependent on the presence of the same lipoidophile antibody in the serum as is responsible for the fixation of complement in the Wassermann reaction.

The Kahn test is the most widely employed and probably the most reliable of all the flocculation tests. As in other flocculation tests, complement is dispensed with in this. Hence, it is simpler and quicker to carry out than the Wassermann test. The

antigen employed is the alcohol soluble, ether insoluble lipoids of the bovine heart. The same antigen reinforced with cholesterol may be used also for the Wassermann test. But, for the precipitation test a stronger concentration is necessary. The quantitative relationship of the antigen with the syphilitic serum would appear to determine the type of the ensuing reaction, precipitation or complement-fixation. For details of the Kahn test refer to books on practical bacteriology.

The Kahn test is probably as sensitive as the Wassermann test. Many workers report that the results of the Kahn and Wassermann tests practically always tally. A serious disadvantage of the test is that doubtful results are difficult to read.

Prophylaxis. There are no means of active or passive immunisation against syphilis. The prevention of syphilis, as in other venereal infections, is through avoidance of promiscuous contact and the development of clean individual and social morals. The immediate local application of antiseptic remedies after exposure is invaluable in prevention. They may be successful even if applied within an hour after exposure. None of the proved therapeutic drugs is prophylactic and perfunctory administration of them after contact with the hope of aborting the infection should be avoided, as it only serves to mask it. General measures for the protection of the individual and the society are early and energetic treatment of cases, rigorous control over prostitution and general education of the public. Early detection of syphilis in the expectant mother followed by thorough treatment is very important.

Therapy. There is no specific treatment for syphilis. Organic preparations of arsenic and bismuth are sovereign remedies, but they are not so efficient in the late as in the early stages. Mercury has been almost entirely replaced by bismuth. Persistent treatment is very essential in all forms of syphilis, especially in late syphilis, regardless of negative serology. Potassium iodide is a valuable adjunct in the late stages. Pyrotherapy has been found useful in neurosyphilis, especially the parenchymatous form. Penicillin has been reported upon very favourably. A total of 2·5 million units constitutes a course. It is too early to assess its permanent value. In view of the fact that some relapses have occurred, combined treatment with penicillin and arsenic has been advocated recently.

With energetic treatment, complete cure is attainable in the early stages, but that may not be easy or even possible in late and latent cases. No reliance should be placed on a single negative serum reaction; safety lies in maintaining persistently negative serum reactions and reactions of the spinal fluid in syphilis of the nervous system.

Treponema Pertenue. *Treponema pertenue*. the cause of yaws or framboesia, was described by Castellani in 1905. It is morphologically indistinguishable from *T. pallidum*. The name pertenue was given to it as at the time of discovery it was considered to be thinner than *T. pallidum*. Monkeys and rabbits have been successfully infected with *T. pertenue*.

It has been suggested that *T. pertenue* is identical with *T. pallidum* and that yaws is only a modified form of syphilis. The results of cross immunity tests in monkeys and rabbits seem to contradict this view, but further investigations have to be done before this point can be cleared.

Yaws is practically confined to the tropics; it is mainly rural in distribution. It is very rampant in many of the Pacific islands like Fiji and Samoa, in Tropical Africa, in the West Indies and to a less extent in India: Assam, in this country, Burma and Ceylon are heavily infected. Though pathologically and clinically yaws presents a striking resemblance to the cutaneous lesions of syphilis, it differs from the latter in its non-sexual mode of transmission. Yaws is highly contagious and all evidence suggests that its mode of spread is mainly through direct contact, the organism entering through open wounds or abrasions. Foetal infection does not occur. Children and young adults are more frequently the victims than others. Natural immunity against yaws does not exist.

The lesions are scattered over the skin. The initial lesion or yaw is a papule which typically develops into a discharging granulomatous eruption, later becoming encrusted. About six to twelve weeks after the primary lesion appears, secondary lesions appear. These have the same general characters as the primary lesion. A tertiary stage similar to that in syphilis has been described by many workers. Internal organs are seldom affected; but involvement of the cardiovascular and central nervous systems has been reported. Discharges from the skin lesions show the presence of the treponemata in large numbers,

especially in the early stages. Their mode of demonstration is similar to that of *T. pallidum*. An antibody-like substance, resembling the syphilitic reagin, apparently develops and so the Wassermann and flocculation tests yield a high percentage of positive results. Organic arsenic and bismuth are likewise effective in the treatment of yaws, again revealing a close kinship of this disease with syphilis.

Leptospira Icterohaemorrhagiae. *L. icterohaemorrhagiae* was discovered by Inada and Ido in 1915 from cases of Weil's disease. It is found naturally in the reservoir host, the wild rat, in the human lesions and for sometime in the moist soil and water contaminated with rat's urine. It seems likely that the parasite can live in water in a free-living state for several months. *L. biflexa*, usually found in water, has been suggested to be the free-living phase of *L. icterohaemorrhagiae*; they are morphologically indistinguishable.

Morphology and Staining. This organism is 7–14 μ by 0.15 μ, with numerous, regular, very fine, primary coils and pointed ends, one or both of which are characteristically recurved (p. 2). The culture forms are longer than the tissue forms; the organism is actively motile with rotatory or wavy movements. Films are stained by Giemsa's or Fontana's method and section by Levaditi's. The closely wound texture is often lost in stained preparations; though the coils are opened out, the terminal curvature is preserved. Multiplication is by binary fission. It seems likely that distinct serological races of *L. icterohaemorragiahe* exist in different regions.

Cultivation is not difficult; it grows readily on Noguchi's, Fletcher's or Schuffner's medium (p. 110). It is an aerobe; some regard it as microaerophilic. The optimum temperature is 25°–30° C., but growth may occur at 37° C. The growth is slow, taking about a week. Cultures remain viable for four to six weeks at room temperature. Bile salts dissolve *L. icterohaemorrhagiae*.

Pathogenesis. *L. icterohaemorrhagiae* causes Weil's disease. Natural infection of dogs and foxes with this parasite has been reported.

Guinea-pigs are very susceptible and rabbits to a much less extent. The intraperitoneal inoculation is the best method of transmitting the infection; the infection can also be induced by rubbing the infectious material on the unshaven skin or the conjunctiva. Following infection, the guinea-pig develops high fever

lasting for about four days when deep jaundice sets in and the animal dies in the course of about ten days. Generalised jaundice, multiple haemorrhages in the lungs and abdominal viscera and enlargement of the spleen are the outstanding features on post-mortem examination. The liver and kidney are both found teeming with leptospirae. During the pre-icteric period the organisms are also found in fairly large numbers in the blood.

Weil's disease, or infectious jaundice, is an acute infectious disease, endemic in many parts of the world and occasionally breaking out in epidemics. It is widely prevalent in Japan. Recently, several small epidemics have been reported from various parts of Europe including Britain. It occurs in some parts of India, especially during the cold weather; its incidence is heavy in the Andamans. Persons coming in contact with stagnant water in the rat-infested areas, labourers working in the damp fields, sewer workers, miners and soldiers in wet trenches are the usual victims. Hence Weil's disease is also an occupational disease. Rats are the reservoir hosts. Up to 40 per cent. of *R. norvegicus* are found infected in different regions. The parasite occurs in the blood and internal organs of the rodent, causing only a mild infection without jaundice. It is excreted in the urine which thus forms the disseminating agent of the infection.

How exactly man acquires the infection, is not clear. It is not through bite. The urine of the infected rats containing the leptospira contaminates the soil and water of the rat-infested localities. It is known that the parasite can remain viable in these situations for relatively long periods. Man may be getting the infection through contact when working in such infested places or when wading or bathing in the infected water, the organism entering through breaks in the skin or mucous membrane. The leptospira can also enter through the intact surface; probably a sodden condition of the skin from continuous wetting may favour penetration. The possibility of infection through contaminated food or water cannot be altogether ruled out. Human infection with *L. canicola*, a natural pathogen of dogs, has been reported; this leptospira is less virulent to guinea-pigs than *L. icterohaemorrhagiae* and is serologically distinct from it.

The incubation period is 5–7 days. This is followed by an initial febrile period with high temperature and severe general disturbance and albuminuria lasting for about six days when

jaundice, usually deep, makes its appearance. The disease may sometimes be mild and unassociated with jaundice. The infection may even be latent. Relapses may occur as in relapsing fever. *L. icterohaemorrhagiae* is highly invasive and is present in the blood during the pre-icteric period of the illness. It may be scanty and difficult to demonstrate, especially towards the end of the febrile period. The leptospirae disappear from the blood with the onset of jaundice. After the first ten days, it begins to be excreted in the urine and may persist in it up to forty or even sixty days. Probably under the influence of bile or acidity, the leptospira present in the urine displays marked morphological aberrations and is often difficult to identify. The organism is present in the internal organs, in large numbers in the liver and kidneys. Its occurrence in the cerebrospinal fluid has been reported. The mortality rate varies up to 30 per cent.

Immunity. Antibodies like agglutinins and lysins are formed early in the disease. They begin to appear in the blood from the fifth day after the onset and progressively increase in titre, reaching a maximum concentration by the fifteenth day. Agglutinin titres up to 1 : 100,000 have been reported. After recovery antibodies persist in the blood for many years. In contradistinction to other spirochaetal infections, recovery from Weil's disease is followed by immunity of a high degree and long duration. Convalescent serum is found to possess protective properties.

Diagnosis. During the first week of the disease, examination of the blood should be carried out under dark field illumination as also after staining by Giemsa's or Fontana's method. The number of organisms in the blood may be few or many. In any case, the earlier the examination, the greater are the chances of success. Failing this, attempt should be made to cultivate the organism in any of the media mentioned under cultivation. The urine after the tenth day may likewise yield positive results by any one of the methods mentioned above. A catheterised specimen should be centrifugalised and the deposit examined. It is worth while remembering that the leptospira in urine may not always exhibit the characteristic morphology, and the close-set primary coils may be difficult to make out even by careful examination.

The pathogenicity test is another important diagnostic procedure. This is done by inoculating the guinea-pig with blood

in the early stage or with urine later; the intraperitoneal is preferable to the subcutaneous route. As the organism may be scanty in the blood, not less than 5 ml. should be injected. The animal develops the disease with high temperature and later intense jaundice and dies in about ten days with the characteristic evidence of the disease (p. 651). The leptospira in the urine often fails to produce the typical disease in the guinea-pig, jaundice being conspicuously absent. The organism may likewise be few in the tissues.

Serological reactions afford a very valuable alternative diagnostic procedure. Even in latent infections, antibodies are produced and serology is the only method of revealing such cases.

The agglutination test is done with the patient's serum after the first five days of the illness. Either the microscopic or the macroscopic method may be followed. As it is probable that there are different serological races of *L. icterohaemorrhagiae*, the use of polyvalent antigen is preferable. A young living culture grown in any leptospira medium, or the same killed with 0·5 per cent. formalin, may be used as the antigen. The general technique of the test is the same as that in the Widal reaction. In the microscopic method, the antigen-antibody mixture should be incubated at 32° C. for two to three hours when a drop of it is removed to a slide and examined under dark ground illumination. When living cultures are employed as antigen, clumping occurs in positive cases in the lower and lysis in the higher dilutions of the serum; the latter phenomenon does not occur with formolised culture. Usually, high titres, 1 : 640 or over, are obtained in positive cases, but, as in all agglutination reactions, a rising titre is the safest criterion. In the macroscopic test, the best results are obtained with an antigen standardised to contain not more than forty organisms per field when examined under dark ground illumination with one-sixth inch objective. After adding the antigen to the serum dilutions, the tubes are incubated for two to four hours at 37° C. Macroscopic agglutination in a dilution of 1 : 40 is suggestive and a higher titre diagnostic. It may be repeated that a rising titre is the surest guide.

The Rieckenberg reaction, also called the adhesion phenomenon or the thrombocytobarin reaction, is another useful diagnostic test (p. 627). It is done by mixing together equal volumes of an young culture of *L. icterohaemorrhagiae*, patient's serum,

Bact. coli or platelets in saline suspension and a 1 : 5 dilution of fresh guinea-pig serum. After incubating the mixture at 37° C. for half an hour, a slide preparation is made from it and examined under dark ground illumination. In positive cases, the bacteria or platelets are found sticking on to the spirochaetes; in controls put up with normal serum, it is absent.

Prophylaxis. No specific prophylaxis is available. The destruction of rats in mines, drains, fish curing yards and similar places, treatment of open wounds and abrasions on individuals, avoidance of suspected water source for bathing or other purposes and similar precautionary measures may reduce the incidence of infection.

Treatment. Convalescent serum is of some value. Immune serum from the horse has been found to produce clinical improvement and reduce the mortality rate to some extent. Chemotherapy has proved ineffective.

Leptospira Hebdomadis. *L. hebdomadis* is the cause of seven-day fever in Japan. Unlike infectious jaundice, seven-day fever is a benign condition, usually causing no jaundice and no mortality. The parasite is distributed in the tissues as in Weil's disease and is present in the blood during the febrile period, after which it is discharged in the urine, may be up to the fortieth day of the disease.

L. hebdomadis is morphologically indistinguishable from *L. icterohaemorrhagiae* but is serologically distinct. It is virulent to young guinea-pigs but does not cause such severe infection as with *L. icterohaemorrhagiae* nor always death. It is a primary pathogen of the field mouse (*Microtus montebelloi*), from which it is excreted in the urine. Man gets the infection probably in the same way as in infectious jaundice.

Various other leptospiral infections with general resemblance to Weil's disease, but much milder, have been reported from different regions. They are ascribed to different species of leptospira: *L. grippo-typhosa* of swamp fever of Eastern Europe, *L. autumnalis* of harvest fever of Japan and *L. pyrogenes* of Sumatra. Further investigations are required to establish that they are separate species.

CHAPTER XXXIV

RICKETTSIA

Rickettsia is the generic name applied to a group of micro-organisms whose systematic position seems to lie between bacteria on the one hand and viruses on the other. The generic name was first applied by Da Rocha-Lima (1916) in honour of Ricketts who was the first to describe these organisms. In their general morphology the rickettsiae resemble the bacteria. But in their non-cultivability in lifeless cell-free media, they differ from bacteria and resemble the viruses. Practically all doubts about their living nature have been set at rest and they are now considered as living things, capable of growth and multiplication. The first one to be described was the rickettsia of Rocky Mountain spotted fever by Ricketts in 1909. Since then, several species, about fifty, have been described, some of which are found in constant association with diseases in man and lower animals and are considered pathogenic.

Ecology. *Rickettsiae* are natural parasites found in the alimentary canal of certain arthropods, such as lice, fleas, ticks, mites and bugs. There they live either extracellularly in the lumen or intracellularly in the epithelial cells lining the canal. They are not known to cause any harm to their natural insect hosts except lice, but some are pathogenic to the mammalian hosts on which these arthropods are ectoparasites. The pathogenic rickettsiae lead an intracellular life whether in the tissues of their animal hosts or in the alimentary tract of their natural arthropod hosts. These insects are the natural transmitters of infection.

Morphology and Staining. Generally, the rickettsiae do not pass through the usual bacterial filters. They are coccoid, diplococcoid or bacillary in form, measuring $0{\cdot}3\text{-}0{\cdot}5 \times 0{\cdot}3$ micron in size. They are very pleomorphic and elements up to 2 microns long may be encountered. They occur singly, in pairs and often in irregular masses. The majority of them are non-motile.

Studies with the electron microscope indicate that in general structure the rickettsiae resemble the bacteria.

They are difficult to stain and when stained exhibit a hazy outline. They are Gram-negative. By Giemsa's method they stain reddish purple and by Castaneda's method they stain blue, thus differing from ordinary bacteria. The coccoid forms are stained uniformly, whereas the bacillary forms often display bipolar staining.

Cultivation. With the exception of *R. melophagi*, a non-pathogenic form, *Rickettsia* has never been cultivated on artificial media free from living cells. But they grow and multiply in tissue culture (tissue-plasma or tissue-Tyrode medium) and in the chorio-allantoic membrane of the developing chick. In these media multiplication of the organism occurs entirely within the host cells which finally die and disintegrate, liberating the rickettsia bodies. Such artificially grown pathogenic rickettsiae maintain their virulence, as can be readily proved by animal inoculation.

Very little is known about their biochemical and metabolic activities. In resistance to bactericidal agents they resemble the vegetative forms of bacteria. They are resistant to drying and glycerine, recalling the behaviour of viruses towards these agents. Outside the host cell rickettsiae do not remain viable for more than a very brief period, unless it be in the excreta of the insect vector.

Immunity. Recovery from an attack of rickettsial disease usually confers solid and lasting immunity. In natural as well as experimental infections agglutinins interacting with the specific rickettsia and with the X strains of *Proteus*, originally isolated from typhus patients, are found to develop. This is the basis of the Weil-Felix reaction. Recently, specific complement-fixing antibodies have been shown to be present in the sera of patients suffering from epidemic typhus. It is reported to be highly species-specific. Protective antibodies also seem to form both as the result of natural infection and of active artificial immunisation.

The Weil-Felix reaction is an apparently non-specific reaction first observed by Weil and Felix in 1915 (p. 480). They found that certain *Proteus* strains, which they isolated from typhus patients and called X 2 and X 19, were agglutinated in the presence of typhus sera in high dilutions, often as high as 1 : 50,000 or more. Subsequently, several more strains have been isolated from cases of epidemic typhus, but they were found

to be much less sensitive. As the antigen concerned with the reaction is the heat stable smooth somatic type, these strains are commonly referred to as OX strains. The H antigen has nothing to do with this reaction. From this the importance of employing the O antigen in the agglutination test is quite manifest.

The production of agglutinins against these proteus strains does not indicate that the latter are in any way aetiologically related to the disease. Nor is there any evidence to sustain the contention that *Rickettsiae* constitute a stage in the life cycle of *Proteus*. As mentioned above, agglutinins against the specific rickettsia are also developed simultaneously with, or rather earlier than, the *Proteus* agglutinins. Absorption tests show that the proteus organisms absorb only the Weil-Felix agglutinins, while the rickettsiae absorb both the Weil-Felix and the rickettsia agglutinins. It is, therefore, highly probable that the reaction depends upon the presence of some common antigenic component in these two groups of organisms. This common substance seems to be a specific polysaccharide hapten. It may be noted that this is a purely random distribution as there is no biological relationship between *Rickettsiae* and *Proteus*.

TABLE XXI

Rickettsial Diseases of Man

Disease	Rickettsia	Insect Vector	Weil-Felix Reaction		
			OX19	OX2	OXK
Epidemic typhus ..	*R. prowazeki*	Louse	+++	+	–
Endemic or murine typhus ..	*R. mooseri*	Rat flea	+++	+	–
Shop typhus					
Toulon fever					
Tsutsugamushi ..	*R. orientalis*	Mite			
Scrub typhus ..	(*R. nipponica*)	larva	–	–	++
Mite typhus of Sumatra ..					
Rocky Mountain spotted fever ..	*R. rickettsi*	Tick	+	+	+
Fiévre boutonneuse					
Sao Paulo typhus					
Indian Tick Typhus					
Trench fever ..	*R. quintana*	Louse	–	–	–
Q fever of Australia ..	*R. burneti*	Tick	–	–	–

Certain other strains of *Proteus* were found to agglutinate with sera from tsutsugamushi patients but not with typhus sera. These were designated OXK strains. The work was soon extended and it was found that sera from the Rocky Mountain spotted fever group of rickettsial diseases agglutinated all the X strains, but only in low titre (see Table XXI). On the basis of this agglutination reaction, therefore, the rickettsial diseases can be classified into three groups: the typhus group, the tsutsugamushi group and the Rocky Mountain spotted fever group.

Pathogenicity. The rickettsiae do not cause disease in their natural arthropod hosts except lice. Experimentally some species are pathogenic to guinea-pigs and monkeys. Following the intraperitoneal injection of infected blood into a guinea-pig, a febrile reaction starts after an incubation period of 5–12 days. Certain species produce a characteristic scrotal reaction, somewhat similar to the Straus reaction, called the *Neill-Mooser Reaction*. Certain cells of the scrotal sac are found crammed with the parasite. The rickettsia bodies are present in the blood. Large number of them are also found in the spleen and brain. The infection is not always fatal to the guinea-pig.

Four species of *Rickettsiae* are pathogenic to man and one to lower animals.

R. prowazeki	..	Typhus fever
R. rickettsi	..	Rocky Mountain spotted fever
R. nipponica (*R. orientalis*)	..	Tsutsugamushi
R. quintana	..	Trench fever
R. ruminantium	..	Heart water of sheep, goats and cattle

Several serologically different types of *Rickettsiae* have been described as causing typhus fever in different regions. Whether they are only serological races or they are distinct species, is still undecided. Bodies resembling rickettsiae have been recently described in trachoma and psittacosis. But their aetiological relationship to these diseases has yet to be proved.

With the exception of trench fever, all the rickettsial diseases resemble one another closely in their method of transmission and clinical course. They are of world wide distribution, but certain types are found predominantly in certain areas, probably depending upon the distribution of the specific vectors and reser-

voir hosts. As indicated above, they are tentatively classified into three groups: the typhus, Rocky Mountain spotted fever and tsutsugamushi groups. The Australian Q fever may have to be added as a fourth group. Trench fever is also classified provisionally as a rickettsial disease (see Table on page 658).

The Typhus Group. This group includes epidemic typhus and endemic typhus. These are also referred to as the European type and the murine type respectively. Tabardillo of Mexico, a louse-borne disease with epidemic characters, is considered to be a form of epidemic typhus, but it is included under endemic typhus by some writers. Brill's disease is considered by some as a mild form of endemic typhus while by many others as a mild form of epidemic typhus. There is now increasing evidence, based on serological studies, to favour the view that Brill's disease and endemic typhus are distinct entities. These questions await final decision.

Typhus. Typhus, also known by several other names, such as epidemic typhus, typhus exanthematicus, gaol fever and camp fever, is an acute infectious disease now accepted to be due to *R. prowazeki*. Poor standard of personal hygiene, general insanitation, overcrowding, poverty and famine conditions are all important predisposing causes. Hence, the disease is mostly found in backward countries, in the lower strata of society and in association with large assemblages of people as occur during war time, in barracks, concentration camps and prisons. Typhus has been associated with all the great wars of history. It is estimated that 315,000 persons died of typhus in Serbia during the Balkan war of 1915. At the close of the 1914–18 war it was prevalent in Poland, Russia and the Balkan States, the recorded deaths due to it running into millions. During the recent war, there was a definite increase in its occurrence in Europe and North Africa. At the present time the endemic foci are China, Afghanistan, Iran, Iraq, Turkey, Russia, the Balkan States, Spain, parts of France and Germany, Central and South Africa and Central and South America. India is free from it, except possibly the extreme north west. The greatest prevalence of typhus is in winter.

Transmission. The transmitting vector is the louse, *P. corporis* and probably also *capitis*. The reservoir host is the sick man and by sucking his blood the louse becomes infected. The para-

sites are found in large numbers in the intestines and the faeces of infected lice. The exact mode of transmission, whether it is through bite or through the agency of excreta rubbed in through the bite puncture or other abrasion by scratching, is not definitely known. In the louse hereditary transmission of infection does not occur. The infected lice usually die in about two weeks. The parasite remains viable with undiminished virulence for many days in the dried faeces of lice. It has been shown that laboratory animals can be infected by the intranasal inoculation of infected material. From these observations it would seem possible that inhalation of infected material may be another method of natural transmission.

The incubation period ranges between 5 and 20 days with an average of 14 days. The fatality rate is high, varying from 20–60 per cent. One attack usually confers substantial immunity, but it may not be lasting. Second attacks are milder. A certain degree of endemic immunity has also been found to exist in endemic areas.

A generalised macular or haemorrhagic rash appears on the fourth to the sixth day. The spleen is moderately enlarged, soft and pale pink in appearance. The essential lesion consists of focal damage of the capillary and pre-capillary vessels, characterised by endothelial proliferation, swelling and necrosis with thrombosis and by the formation of perivascular nodules caused by the exudation of lymphocytes, plasma cells and monocytes. The commonest sites of these lesions are the brain, skin, heart, kidneys, adrenals, testes and epididymes.

During the febrile period the parasites are present in the blood where they seem to remain closely adherent to the red cells. They are also present in the vascular lesions and in the spleen, brain and other organs. They can be demonstrated microscopically in the endothelial cells lining the serous cavities and in the vascular endothelium in the characteristic vascular lesions described above. But their presence can be more easily established by animal experiments. The disease is transferable to monkeys and guinea-pigs. Blood drawn at the height of the illness and inoculated intraperitoneally into guinea-pigs produces fever in the animals after an incubation period of about nine days. The febrile reaction continues for about five to eight days, during which the blood of the animal is infective to fresh guinea-pigs or

monkeys. Recovery is the rule and the animal is resistant to a second infection. By similar experiments, the spleen, brain and other organs can be shown to contain the organism. Rickettsia-free louse, experimentally fed on the patient's blood, develops the parasite in the lining epithelial cells of the intestine and becomes infective to monkeys and guinea-pigs in four to five days after the feed. In the absence of such development of the parasite, the louse does not become infective to other animals. It may be noted that all lice fed on typhus cases do not become infected; a fair proportion, less than half, escapes infection.

Diagnosis. The Weil-Felix reaction is a valuable diagnostic test (p. 657). But it does not serve to differentiate typhus from Rocky mountain spotted fever, as a positive reaction is obtained to the same antigen in the latter disease also. The agglutination reaction using the specific rickettsia has not been developed as a routine measure. It may be of assistance in the differential diagnosis. The Weil-Felix reaction usually becomes positive by the end of the first week of fever, the positivity reaching its height towards the close of the illness and thereafter vanishing rather rapidly. As in the case of the Widal test, it should be repeated and a rising titre given greater value. The test is performed in the same manner as the Widal test but using a suspension of OX 19 as the antigen instead of the enteric antigen. OX 2 is less sensitive. A live culture is more satisfactory. Typhus sera often give a titre between 1 : 10,000 and 1 : 100,000. It should be borne in mind that a titre of 1 : 320 may be given by other diseases causally unrelated with the rickettsia organisms. Some workers, however, consider that a titre of 1 : 100 or above is diagnostic. It is of importance to remember that an anamnestic response of *Proteus* agglutinins occur in many febrile conditions, particularly in typhoid fever. Whereas in the latter the titre of its agglutinins continues to rise, that of the former does not do so after the first week.

Recently, it has been shown that a positive complement-fixation test is obtainable with the sera of typhus cases. A positive test is obtained in the second week and remains so for a very long time. That this serves to differentiate typhus fever from Rocky Mountain spotted fever, may turn out to be of great practical value.

Prophylaxis. The prevention of typhus rests on two factors: the control of lice and vaccination. Towards control of lice al

patients and their contacts and all persons coming from typhus infected areas should be completely deloused. Their clothing and bedding must be thoroughly disinfected by the employment of heat, pediculicide powders or chemicals. D.D.T. has been very successfully used during the recent war in controlling epidemics of typhus.

Immunisation against typhus has been done with attenuated rickettsia. But it is risky. The vaccine now employed is dead rickettsia, killed by formaldehyde or phenol. In the Weigl's method rickettsia is obtained by the intrarectal cultivation of the parasite in lice. Obviously, this type of vaccine cannot be prepared on any large scale. Vaccine prepared from about two hundred lice is necessary to immunise one person. This vaccine seems to yield good results. A second source of rickettsia for the preparation of vaccine is from the growth obtained in the yolk sac of the developing chick embryo (Cox's vaccine). Another source is from the lung of rabbits, rats, or mice infected intranasally with the organism. Three intitial doses, seven to ten days apart, followed by a single dose every four to six months are recommended. The degree of protection that vaccines confer and its duration are both not well known. It may not entirely prevent infection but modifies the course of the disease considerably.

Endemic Typhus. The name *R. mooseri* has been given to the organism of endemic typhus. It is probably only a variety of *R. prowazeki.* The reservoir host of the parasite is the rat. Due to this association the disease is also referred to as murine typhus. Brill's disease is considered by some as a mild form of it. Rat fleas (*X. cheopis*) and rat lice transmit the infection from rat to rat. The salivary glands of the infected fleas do not show the parasite. Transmission to man is probably through the agency of infected faeces. Once infection is established in man, lice can transmit it and epidemics of louse-borne murine typhus have been reported from Mexico. Generally speaking, endemic typhus is clinically less severe than epidemic typhus. It would seem that the virulence of the parasite has suffered by its sojourn in the flea host as compared to that of the louse-borne strain. With this parasite the Neill-Mooser reaction is obtained in the male guinea-pig. When the animal is inoculated intraperitoneally with the infected material, it is followed by an inflammatory

reaction of the scrotal sac which shows large number of parasites in the endothelial cells. *R. prowazeki* does not give rise to any such reaction.

Endemic typhus was first described in the coastal towns of the United States. Subsequently, it has been found to have a world wide distribution, preferring coastal to inland areas. Toulon fever and the urban typhus of Malaya are identical with it. The greatest prevalence of endemic typhus is during summer. As in epidemic typhus, the Weil-Felix reaction is positive in high titres and so seldom serves to differentiate between these two forms of the disease. Recent investigations tend to show that agglutination test done with the specific rickettsia may be employed for differential diagnosis. Prevention is by rat control.

Rocky Mountain Spotted Fever. The causative agent is *R. rickettsi.* It was in this disease that rickettsia bodies were first detected by Ricketts in 1909. Ticks are the transmitting vectors; *Dermacentor andersoni* and *Dermacentor variabilis* are the more common transmitters. Hereditary transmission occurs in ticks. The natural reservoir of infection remains undefined. In Brazil the disease is called Sao Paulo typhus.

Agglutinins for OX 2 occur more frequently in this disease than in epidemic typhus. When agglutinins for both OX 19 and OX 2 develop, those for the former show higher titres than those for the latter. Agglutinins for OXK are also said to be formed in this disease but in low titres. The complement-fixation test using the specific rickettsia as antigen gives a positive reaction in the second week of the disease. The Neill-Mooser reaction is said to be positive in spotted fever.

No specific treatment has been so far successful. Preventive methods should be aimed at the eradication of ticks. Prophylactic vaccination is still in the experimental stage.

Boutonneuse or Marseiles Fever. This is another rickettsial fever and is closely related to Rocky Mountain spotted fever. The term *R. conori* has been given to the causative rickettsia; probably it is only a variety of *R. rickettsi.* The transmitting agent is the dog tick, *Rhipicephalus sanguineus.* The disease is common in the Mediterranean regions, Kenya and in some parts of South Africa. The Weil-Felix reaction gives a weak positive for both OX 19 and OX 2 strains. Specific agglutinins are said to develop late in the disease.

Indian Tick Typhus. A typhus-like disease has been reported from several parts of this country—the Kumaon Hills, the Simla Hills, Meerut, Bengal, Assam, Madras, Bangalore and others. Its epidemiology has not been worked out. The vector is unknown; some believe it to be a tick. The disease is probably related to Rocky Mountain spotted fever. The agglutination test with OXK has been reported to be positive from some of these places.

Tsutsugamushi. The causative organism of this disease is *R. nipponica.* At one time tsutsugamushi was thought to be confined to Japan. It is probably identical with the scrub typhus of Malaya, the mite typhus of Queensland in Australia and the pseudotyphus of Sumatra. It is also prevalent in the Philippines and other islands of the Pacific. The natural reservoir of infection is the field vole (*Microtus montebelloi*) and other rodents. From them *Leptus akamushi*, the larval form of the mite *Trombicula akamushi*, conveys the infection to man. The adult mite in Malaya and Sumatra is *T. deliensis.* The adult mites do not feed on animals, but their larval forms are ectoparasites on rodents. There is probably transmission of infection from the infected larva through its subsequent stages to the progeny.

The agglutination reaction with OXK gives a positive of 1 : 160 or more usually by the tenth day, reaching the peak titre by about the commencement of convalescence. The reaction is negative with OX 19 and OX 2. There is no specific treatment for the disease. Preventive measures are directed towards keeping off mites and their larvae. No prophylactic vaccine has been developed against tsutsugamushi.

Q Fever of Australia. It is of recent discovery. The organism responsible for it has been termed *R. burneti.* The tick seems to be the transmitting agent and the bandicoot the animal reservoir. Hereditary transmission probably occurs. The organism is readily filterable through the ordinary bacterial filters, thus differing from other known species of pathogenic rickettsias. Another difference is that no agglutinins for any of the X types of *Proteus* are found in the blood of Q fever patients.

Trench Fever. Trench Fever, or Wolhynian fever, is another disease generally considered to be of rickettsial origin. Attention was first drawn to it during the 1914–18 war, but due to its disappearance after it, there was no opportunity to study its aetiology

and epidemiology. It appeared again in the recent war among the German army in Russia. Transmission from man to man is by the body louse. Whether it is through bite or by excretal contamination, is not definitely known. Lice fed on the blood of patients have been found to develop extracellular rickettsias in their gut. Rickettsiae are also present in the faeces of such lice. They are present in the urine of patients.

Bartonella Bacilliformis

Bartonella bacilliformis causes oroya fever, a disease prevalent in Peru, Columbia and Ecuador. The taxonomic position of it still remains undetermined. From the intracellular position of the parasite some workers are inclined to regard it as closely related to *Rickettsiae*.

The chief features of the disease are intermittent fever, intense anaemia of the megaloblastic type and a high rate of fatality. The parasite is found in the blood invading the red cells and may be demonstrated in blood smears stained by the Giemsa technique. Noguchi has established the causal relationship of *Bartonella bacilliformis* to another clinically dissimilar disease, verruga peruviana, characterised by a nodular eruption. In the tissue macrophages of the lesion the organism is found in large numbers. He has proved the serological identity of the organisms isolated from these two apparently distinct disease entities. Sandflies, *Phlebotomus verrucarum* and *Phlebotomus noguchii*, serve as transmitting vectors of infection.

Bartonella bacilliformis is a rod-shaped pleomorphic organism, 0·2–0·5 micron × 0·3–2·5 microns in size, occurring singly, in end to end pairs and short chains. Coccoid and dumbbell forms also occur. It is actively motile and Gram-negative. By Giemsa's method it stains reddish violet with an indistinct outline and sometimes with a reddish purple granule at one end. It has been cultivated in a semi-solid medium containing serum and haemoglobin and also on solid media containing horse blood. It seems to require for growth the X factor but not the V factor. It is aerobic. The optimum temperature is 25°–28° C. It can survive long in artificial media.

The organism is infective to monkeys. Intravenous inoculation with the infected material results in the production of an identical disease as oroya fever, but without anaemia. The blood

of the animal contains large number of organisms. The nodular form can also be reproduced by subcutaneous inoculation of the material into the eyebrows of monkeys. Numerous organisms are found in such experimental lesions.

Recovery from either form of the disease is said to confer lasting immunity. It has been reported that agglutinins are present in the blood in the early stages of the disease, although they disappear from it soon.

Bartonella muris, a similar organism, appears to cause a latent infection in rats. The occurrence of this is of some practical importance in rat experiments. Splenectomy brings out the infection and the red cells are seen infected. The spleen probably keeps them under check. *Bartonella canis* is said to cause an anaemic infection in dogs.

CHAPTER XXXV

FILTERABLE VIRUSES

Viruses, or filterable viruses, are the smallest known infective agents that cause disease in man, animals or plants. Like bacteria, they have the capacity to invade, grow and multiply in the living tissues and in so doing incite the production of immunity. Viruses exhibit some of the essential attributes of life and are regarded as living things. They show the power of metabolic assimilation, reproduction and reaction to changes of environment and adaptation. In the possession of a definite antigenic make-up with chemical specificity they resemble the bacteria. Taxonomically, they occupy a position lower than that of the rickettsias. As a class, they differ from bacteria in certain respects. They readily pass through filters that hold back bacteria and so the term filterable is applied to them. Unlike bacteria, viruses are too minute to be seen distinctly by ordinary microscopy and so they are often referred to as ultramicroscopic. They do not grow on any culture media usually employed for the cultivation of bacteria but require the presence of living cells for growth. For the above reasons, their existence has to be recognised by the effect that they produce in susceptible animals. These peculiarities also render their study far more difficult than that of bacteria. Neither the microscopic nor the ordinary cultural procedures can help us much. Consequently, our knowledge about them remains very meagre. The position of bacteriophage is unsettled. In point of size it is akin to the virus; it is probably a virus parasitising bacteria.

Other distinctive features are their intimate relationship with the host cells and the formation of inclusion bodies in the affected cells. Viruses also exhibit a marked affinity for certain tissues. They are highly invasive, provoking little or no reaction at the site of entry. None of the viruses synthesises any exotoxin. Not infrequently, they confer on the surviving hosts more solid

and lasting immunity than bacteria. Resistance to glycerine is another characteristic feature of most of the viruses.

The earliest observation that a filterable virus may cause disease was that of Iwanowski who, during his investigation into the aetiology of the mosaic disease of the tobacco plant, demonstrated (1892) that a filter-passing agent was responsible for that disease. This was confirmed by Beijerinck (1898). Next, Loeffler and Frosch (1898) by their crucial experiment showed that bacteria-free filtrate from the vesicular fluid of foot-and-mouth disease of cattle reproduced the disease on experimental inoculation into these animals. Since then, the causal relationship of a large group of infectious diseases of man, lower animals, insects and plants with specific viruses has been established.

Filtration. All the earliest observations on viruses were made with filtrates of pathological materials passed through bacterial filters and filterability, therefore, came to be regarded as an important criterion. Then it was found that some of the viruses, like those of varicella and herpes zoster, were retained by the filters, although they exhibited all the group characteristics of viruses. The list of such non-filter-passing viruses is increasing with more careful studies with improved methods. On the contrary, the mere passage through a bacterial filter does not always justify the term filterable virus. For instance, certain minute bacteria and flexible slender spirochaetes also filter through bacterial filters. For these reasons, the term filterable virus is not quite accurate.

The conditions of filtration have an important bearing on filterability. The filters commonly employed are the Berkefeld N and W, Chamberland L_2 and L_3 and Seitz E.K. types (p. 149). Though their pore size is in the neighbourhood of 3 microns, they retain all organisms above 0·25 micron in size. Clearly then, filtration is not a simple mechanical process, depending only on the size of the pores and the size of the organisms. But it is profoundly influenced by various other factors, such as the composition and the electrical charge of the substance of the filter, the electrical charge of the material to be filtered, plasticity of the organisms, presence and concentration of any protein matter in the filtration fluid, the reaction of the fluid and the temperature, pressure and duration of filtration. The ordinary filters usually consist of positively charged cations of magnesium

or calcium and negatively charged silicon anions. Similarly, viruses carry a negative charge and their filterability is in some degree affected by these relative charges. The virus may be adsorbed by the filter material or by the proteins present in the fluid, thus preventing or retarding filtration; the lower the pH of the solution, the greater the chances of this happening. The test for filterability should take cognizance of all these factors, and the mere inability to pass through a filter should not by itself be regarded as sufficient indication of the size. An inevitable corollary from the above facts is that unless uniformity in technique is carefully observed, results obtained are bound to be divergent.

The Nature of the Virus. The exact nature of the virus has been a subject of considerable dispute, mostly centering on whether it is animate or inanimate. An earlier view is that they are enzymes. Another is that they are autocatalysts, formed afresh every time they act on living cells. This is probably the case with certain viruses pathogenic to plants, for example the virus of the mosaic disease of tobacco, which has been isolated in crystalline form. Most observers now agree that in general the viruses are living things like bacteria capable of self-propagation and in many cases of pathogenicity. As pointed out above, this may not be true of all viruses.

One great difficulty in accepting viruses as forms of life is their extreme smallness in size, the smallest ones approaching the size of the more complex protein molecules. But we have no knowledge of what minimum space is required for the manifestation of life and the carrying on of life activities. But we know that parasitism enables considerable curtailment of life activities. It may be that the viruses are highly parasitic forms depending on the host for most of their life functions, and that only the minimum activity required for the sustenance of life survives in them. When we survey the vast range in size among living things, it should not be difficult to conceive that there can be forms of life yet smaller than bacteria. The mere minuteness of the physical basis of life then should not be a stumbling block in this realisation. It is not merely speculation; by improved methods of investigation most of the objections raised against their living nature have been met and the rival theories in the field proved to be far less tenable.

The suggestion has been put forward that viruses are degraded minute variants of bacteria with all fermentative activities, necessary for carrying out the life functions, lost and retaining only the self-generating capacity essential for the preservation of life. There is but scanty evidence in support of this suggestion. Whether the viruses are degraded bacteria or distinct forms of life, their intimate and obligate relationship with the host cells indicates that their's is a highly parasitic state in which they depend almost entirely on other living cells for their maintenance.

Classification. No generally accepted classification of viruses exists. Any satisfactory scheme must rest on the intimate nature and properties of the virus. Such definite data are lacking, and so any classification based on the present imperfect knowledge is of necessity tentative. Several schemes have been suggested based on different criteria, such as the tissue affinity, the type of the lesion produced and the occurrence of inclusion bodies. Terms such as the neurotropic, dermotropic and the viscerotropic are based on the selective tissue affinity of the respective viruses and are useful only as a working arrangement.

Habitat. Viruses are widely distributed in nature. They are essentially parasitic and are found in the lesions they produce in different hosts—man, animals and plants. They are almost always associated with the tissue cells of the affected parts. They may also exist in the carrier state. The existence of saprophytic viruses and of avirulent variants of the pathogenic ones has been suggested.

Morphology. Our knowledge about the morphology of viruses is very meagre. What information is available on this point, has been gained from a study of their photographs. A few viruses, *e.g.* the ectromelia virus and the fowlpox virus, are coccoid or diplococcoid. Recent studies with the electron microscope tend to show that the vaccinia virus is rectangular like a brick and the tobacco mosaic virus rod-shaped. Limited evidence indicates that reproduction occurs by binary fission.

Viruses are much smaller than bacteria; they are in general too tiny to be resolved even by the most powerful microscope using white light. But the term ultramicroscopic is not free from objection. The larger viruses at least can be perceived as bright spots under dark field illumination. Again, by the compound microscope, using ordinary light and apochromatic

objectives of the highest quality, it is possible to extend the range of visibility and see minute objects down to 74 millimicrons in size, though it is not possible to resolve them and reveal their exact form or structure; they are merely seen. Further, by deep staining, objects slightly gain in size and the larger viruses may thus be brought within the range of distinct vision through ordinary microscope. The above observations also serve to show the inaccuracy of applying the term ultramicroscopic to viruses.

The unit of measurement for viruses is the millimicron or one-thousandth of a micron ($m\mu$ or 10^{-9} metre). The size of most of the viruses lies between 8 and 200 (0·2 micron) millimicrons (Table XXII). The foot-and-mouth virus, measuring 8–12 millimicrons, is the smallest known virus and is very near in size to the serum globulins, measuring about 7 millimicrons. The largest virus known is that of psittacosis with a measurement of 200–300 millimicrons.

The size of viruses is determined by any of the three methods —ultraviolet and electron microscopy, high speed centrifugalisation, ultrafiltration through gradacol membranes of variable pore size (Elford). These different methods give practically identical results and the size of any individual virus as ascertained by any one of them is the same as given by any other.

It has been pointed out under microscopy that in practice the limit of microscopic resolution using visible rays is reached with a particulate diameter of 0·25 micron (250 millimicrons). But the resolving power can be improved by using rays with shorter wave length. Far lower limits of resolution have thus been practically achieved in the ultramicroscope employing ultraviolet rays (p. 56). But these rays do not activate the retina, besides being injurious to it. Hence, in ultraviolet microscopy, photographs of images are taken for purpose of study. Objects as small as 75 millimicrons have been resolved and their photographs taken by this method. From these photographs the size of those objects can be calculated with great accuracy.

Recent advances in electron microscopy (p. 57) have brought within practical bounds the photographing of even the smallest virus known; and further advances in our knowledge about ultramicroscopic objects may be confidently expected in the near future.

TABLE XXII

The Approximate Size of Viruses

Viruses, etc.	Size in $m\mu$
Staphylococcus (for comparison) ..	1,000
Psittacosis	200–300
Smallpox } Vaccinia }	125–175
Lymhogranuloma inguinale ..	125–175
Herpes febrilis	100–150
Rabies	100–150
Influenza	80–120
Acute lymphocytic chorio-meningitis ..	37–60
Rift Valley fever	30
St. Louis encephalitis	25
Yellow fever	18–28
Poliomyelitis	12–17
Foot-and-mouth disease	8–12
Bacteriophage	8–75
Serum pseudoglobulin	7 (approximately)
Oxyhaemoglobin	5
Egg Albumin	4

Another method of estimating the size of viruses is by centrifugalisation at very high speed, such as 60,000 r.p.m. (ultra-centrifugation). Owing to the mechanical difficulties of constructing them, such machines are very costly. The rate of sedimentation of suspended particles is influenced in a definite manner by several factors, one of which is the size of the particles. The values of the other factors can be determined and from these the diameter of the particles calculated.

The third method of determining the size of viral bodies is by filtration through collodion membranes of graded pore size, called *gradocol* membranes (p. 153). Earlier studies with collodion membranes gave conflicting results owing to the imperfections in the preparation of collodion filters, but recent improvements have resulted in the production of membranes of great fineness and accurate pore size and the results of measurement of the size of viruses by these closely agree with those obtained

by other methods. By this technique, viruses as small as 8–12 millimicrons can be measured with considerable precision.

Cultivation. Viruses do not grow on ordinary lifeless media; they grow, however, in the presence of living cells. Vaccinia virus was the first to be cultivated; it was by the in vivo method in the rabbit. Next, it was successfully cultured in vitro in a medium composed of rabbit kidney and plasma. The presence of actively growing cells of tissue culture was then considered necessary for growth. Later, it was shown that proliferating cells were not indispensable for its growth when the successful cultivation of the vaccinia virus was accomplished in a medium containing minced fresh hen or rabbit kidney and fresh serum mixed with Tyrode's solution (Maitlands). In this medium there is no proliferation of tissue cells, but their viability is maintained for some days. The cultivation is done in specially designed containers called Carrel flasks. Many more viruses have since been cultivated by this method. But owing to the difficulty of cultivation, a common method of maintaining viruses in the laboratory is by serial passage through susceptible animals.

Further investigations showed that chorio-allantoic membrane of the developing chick embryo was a more suitable substrate for the cultivation of viruses. Eggs, ten to twelve days old, gave the best results. The manipulative technique requires dexterity and considerable aseptic care. After spotting out the position of the membrane by transillumination in the dark, a window is made in the shell and the material is inoculated into the membrane. The flap of the shell is carefully replaced and sealed with melted paraffin. The egg is then incubated at 37° C. for two or three days or longer. Evidence of growth is found in the form of opacities and thickening of the membrane. Further proof is adduced by dissecting out and grinding up the membrane in sterile saline and testing for infectivity on suitable animals. In positive cases the saline suspension will be found to contain large doses of the virus, supplying proof of multiplication and growth. This is relatively a simple method and many of the viruses can be grown by it. A more recent discovery of some importance is that the influenza virus grows well in the allantoic cavity. However, the exact growth requirements of viruses still remain obscure and so far no virus has been grown in the absence of living cells.

Resistance. Generally, viruses resemble the vegetative bacteria in their resistance to destructive agents. They are probably slightly more resistant to chemicals than the non-sporing forms of bacteria. It is to be noted that resistance tests with most viruses have been conducted not in their pure state but with crude suspensions. Sunlight, heat and oxidising agents prove rapidly lethal to them. Viruses withstand cold and desiccation for long. They may be frozen, dried and stored with little inactivation. In contrast to the bacteria, they display marked resistance to glycerol and one of the methods of preserving the virus is in 50 per cent. pure glycerol in saline at 4° C. In this state the Poliomyelitis virus has been found to remain viable for eight years. Unlike bacteria, viruses do not seem to be affected by repeated freezing and thawing.

Metabolism. Very little is known about the metabolic activities of the virus. The question is complicated by the fact that viruses do not grow independently.

Chemical Composition. Our knowledge about the chemical structure of the virus is very limited. The difficulty of getting them in sufficient quantity and in a state of relative purity has prevented the study of its chemical constitution. Chemically, viruses may not materially differ from bacteria. The results of investigations with the vaccinia virus are in harmony with this suggestion; this virus are complex in its structure, containing proteins (nucleoprotein), carbohydrates and fats. From the tobacco mosaic virus an apparently pure protein, with chemical characters of nucleoprotein, has been prepared. It is of high molecular weight, a so-called "heavy" protein. Even after going through the physical and chemical processes of preparation, it retains the virus activity and so it is now considered to be identical with the virus. It has been prepared in a crystalline form.

Antigenic Structure. Not much is known about the antigenic structure of viruses. Fundamentally, there may not be any difference in this respect from bacteria. Certain viruses have been shown to possess a complex antigenic constitution like bacteria, exhibiting serologically distinct types of antigens, *e.g.* the foot-and-mouth virus. There is also some evidence to show that common antigenic components exist among members of the same virus group, thus evincing group specificity as among allied bacteria. The occurrence of polysaccharide haptens in

certain viruses, for example the vaccinia virus, once again illustrates a close similarity in the general pattern of the antigenic make-up between viruses and bacteria.

Immunity. Apparently, the mechanism of immunity against virus infections is essentially the same as that against bacterial infections. The same factors are, in all likelihood, concerned with the production of resistance in both. Man is naturally immune from certain virus infections which affect animals and plants. In natural diseases caused by viruses, antibodies are developed in much the same way apparently as in bacterial infections. Such immune factors are also produced in experimental animals but efficiently only in the same species. Recovery from virus infections confers on the host a more substantial and lasting immunity than in the case of bacterial infections. The transfer of passive immunity may occur in utero and is possible artificially. Rarely viruses may also induce allergic sensitisation (*vide infra*).

As mentioned above, in many virus infections recovery from an attack usually confers solid and lasting immunity, for example measles, yellow fever and smallpox. In this respect viruses differ somewhat from bacteria. In some diseases, however, the immunity is short-lived as illustrated by common cold, influenza and herpes febrilis. Immunity is frequently found to be associated with the presence of circulating antibodies. Such antibodies are also developed in experimental animals and after active artificial immunisation. Their exact nature is not known, but in their mode of action they resemble the bacterial antibodies. The serum containing such antibodies displays the property of neutralising the specific virus in vitro and presumably in vivo, hence the name *protective* or *neutralising antibodies* (p. 238). Their presence can be demonstrated by the *neutralisation test* on suitable animals. These are injected with mixtures of the virus and the serum and with the virus without the serum for control. The control animals develop the characteristic lesions, whereas the former escape if the serum contains the specific antibodies. Probably, these antibodies act by inactivating the antigen after combining with it. The naturalisation test is the most commonly employed serological reaction in the study of virus diseases.

In some of the virus diseases at least, agglutinins, precipitins and complement-fixing antibodies are formed by the host tissues in a specific manner and are present in the blood. Their presence

can be demonstrated by appropriate reactions. Smallpox, herpes, influenza and psittacosis are examples of such diseases.

The production of allergic hypersensitisation of tissues is not common with viruses. In a few cases, as lymphogranuloma inguinale and psittacosis, a hypersensitive state is established, an observation of some practical value in diagnosis. On the basis of this, a valuable skin test, the Frei test, has been developed for the diagnosis of the former.

How far the humoral antibodies are able to help the body in combating infection, is not clear. It may vary from disease to disease. The intracellular position of the virus protects it from the action of the circulating antibodies. In other words, when a virus has established itself in the cell, antibodies are not very effective against it; they may serve to prevent further spread.

Attempt to induce active immunity against virus infections was the logical outcome of our knowledge of the nature of viral immunity. In fact, active immunisation was in vogue long before anything was known about the nature of the complex mechanism of immunity. Vaccination against smallpox dates back from ancient times and anti-rabic vaccination has been in practice for several decades. Similarly, passive immunisation is also possible; convalescent sera are useful in many cases for cure and prevention. Antiviral sera produced in animals are not very potent.

The method of inducing immunity may be by injecting with virulent virus, attenuated virus or with virus treated with bactericidal agents, like phenol and formalin. Because of the inherent danger, the first method is usually employed only in animals and then under the protection of antiserum given separately or mixed with the virus, for example immunisation of hog against hog-cholera. The serum covers the immediate risk from the virus. Smallpox vaccination is an example of immunisation with attenuated live virus; the vaccine in this case does not contain any virucidal agent. Similarly, in anti-rabic immunisation the virus used is one attenuated for man, by passage through rabbits; the vaccine is usually a carbolised one.

Toxin Production and Pathogenicity. Viruses do not produce any extracellular toxin and the actual toxic agent concerned in the production of disease is presumably the viral body itself.

Viruses exhibit varying degrees of pathogenicity and virulence.

Great infectiousness is a marked feature of certain viruses; the variola virus is an example of this. Some viruses are markedly virulent; for example, extremely minute doses of the yellow fever virus can transmit the infection to fresh host. Inapparent infection and atypical disease may both occur. These are of great importance in epidemiology, as they present serious problems in the control of infections. In virus infections a certain degree of endemic immunity, established as the result of long contact, may modify the disease course in a community in which such infections are endemic, as for example yellow fever.

The virulence of viruses can be altered as in the case of bacteria (p. 202). For example, the variola virus undergoes considerable modification as the result of serial inoculation into calves and on reinoculation into man produces vaccinia and not variola. The conversion of street virus into fixed virus by serial passage through rabbits and of the viscerotropic yellow fever virus into the neurotropic brain-fixed virus in the mouse are other examples of such adaptive modifications. These provide further proof that viruses display host specificity as well as selective tissue affinity. Certain virus infections occur only in man and even their experimental reproduction in animals is difficult or impossible to achieve. On the contrary, many of the virus diseases of animals do not affect man. Further, among animals viruses pathogenic to one species are not always pathogenic to other species. Viruses also show, in a greater degree than bacteria, tissue selection and localisation. The lesions in variola and varicella are mostly confined to the skin; the tissue primarily affected in rabies, poliomyelitis and herpes is the nervous system; the viruses of influenza, measles and common cold affect the respiratory tract; the yellow fever virus and the virus of infective hepatitis attack the viscera.

The application of Koch's postulates to prove the aetiological relationship of virus diseases is fraught with far greater difficulty than in the case of bacterial infections. That the existence of a virus can be proved only by the results of animal inoculation, imposes a serious handicap. Another obstacle is that no susceptible animal species for experimentation have been discovered for all the viruses. It is important to bear in mind that natural virus infections are more frequent in experimental animals than natural bacterial infections and that it is far more difficult to detect the former than the latter. Otherwise, fallacies will creep in easily.

The serial passage through susceptible animals, reproducing the condition every time, is the surest way of establishing the causal relationship of a virus with the specific disease. Where man only is susceptible and all experimental animals are refractory, the proof of the aetiological role of the virus is sought for from other, though less certain, evidence.

Viruses cause a large number of infectious diseases in man, varying widely in acuteness and clinical manifestations. A few of them are responsible for some of the severest epidemic diseases which take a heavy toll of human life. Smallpox, influenza and yellow fever are the outstanding examples in this category. Viruses also cause many important diseases of animals, causing great economic dislocation and loss.

The mode of transmission of infection in virus diseases also varies as in the case of bacterial infections. It may happen in any one of the following ways: direct contact and implantation of the virus from man to man as in lymphogranuloma inguinale or from animal host to man as in rabies; direct deposition of the contagious discharges on skin or mucous surfaces; air-borne infection and entrance *via* the respiratory tract through droplet or dust as in influenza, measles, herpes, variola and so on; and inoculation through the skin by insect vectors, *e.g.* yellow fever, dengue and phlebotomus fever. The ingestion of infected food and water may also serve as mode of spread. Rarely, in utero transmission of virus infection may occur; a variolous pregnant woman, for instance, may transmit infection to the foetus. Carriers apparently exist, playing a role in the propagation of infection.

Mention has been made previously that growth and multiplication of the virus take place within the tissue cells and that its occurrence independently of living cells is not known. How exactly the growing virus damages the cells, is not clear, but proliferative and inflammatory changes supervene soon after invasion, resulting in the death and destruction of tissues. An abnormal phenomenon encountered in some of the virus diseases, and not seen in any bacterial infection, is the presence of certain peculiar structures within the affected cells; these are called *inclusion bodies*.

Inclusion Bodies. Inclusion bodies do not appear in all virus diseases. They have different appearances in different

diseases and in the same disease they may not look alike in different host species. The same virus may form these bodies only in certain tissues and not in others; for example, the cell inclusions in variola and wart are formed only in the epidermis and not in the corium. Inclusion bodies are not seen at all stages of the same disease; for instance, the intranuclear inclusion bodies in warts are present only in the earliest stages of the condition and not in the later.

Inclusion bodies may also develop in tissue culture. Extracts of tissues containing cell inclusions as well as filtrates from such tissues incite the production of inclusion bodies. They are also formed in experimental lesions produced with the living virus. Dead virus, though immunising, fails to stimulate their formation.

Inclusion bodies are cytoplasmic or intranuclear in position. Examples of the former are the Negri body of rabies, the Guarnieri body of variola and vaccinia and the Bollinger body of fowlpox and of the latter are those found in herpes febrilis, varicella, yellow fever and poliomyelitis. The inclusion bodies may take any shape and size within limits. In structure they may be hyaline or granular, homogeneous or irregular. Both Mann's and Giemsa's stains are useful for staining them (p. 79). They stain eosinophilic or basophilic. They may also show mixed staining, the ground substance taking one stain, the elementary corpuscles another.

Nothing definite is known regarding the nature of inclusion bodies. What evidence is available, is in favour of the view that they are colonies of virus held together by an amorphous lipoidal matrix. It may be that inclusion bodies are specific and that they are associated with the infectivity of the tissues wherein they occur. In one case at least, the question has been practically settled: a single Bollinger body of fowlpox, washed free of all extraneous matter, is found capable of causing infection. It seems more than possible that the elementary bodies are the ultimate basic units constituting all types of inclusion bodies and that they are the actual virus (*vide infra*). But more work is required to prove the truth of these.

An earlier view was that inclusion bodies consisted of aggregates of the infective agent surrounded by a veil or mantle formed from the invaded cell substance; hence the name *Chlamydozoa*

(Chlamydos, mantle; zoon, an animal) was proposed to them. It has been suggested that the inclusion bodies are protozoal in nature and represent a stage in the life cycle of the parasite. Again, some regard them as mere degeneration products of cells.

However, the presence of cell inclusions is usually regarded as sufficient evidence of infection. But their great importance lies in the practical value in diagnosis. The finding of Negri bodies in the appropriate tissue, for example, has been recognised as proof of rabies even before the viral aetiology of the disease was accepted, and their demonstration has been a routine diagnostic procedure ever since. It may, however, be noted that the appearance of intranuclear inclusion bodies is not necessarily indicative of virus infection. Their occurrence has been noted in other conditions as well. For instance, bodies similar to the intranuclear inclusion bodies of yellow fever occur in the liver cells in severe burns.

Elementary bodies are extremely minute bodies occurring in the cells and exudates from infected tissues. They were first noted by Borrel in fowlpox and later by Paschen in smallpox. Their presence has since been demonstrated in vaccinia, varicella, herpes zoster, psittacosis, molluscum contagiosum and others. Elementary bodies can be stained by Paschen's, Castaneda's or Giemsa's stain; they appear red or purplish. In the stained state, they can be resolved by the ordinary microscope as they become apparently larger through accretion of the dye particles. Thus, Paschen bodies when stained appear spherical in shape like cocci and measure about 0·2 micron. By microtechnique Bollinger bodies have been shown to be collections of Borrel bodies which are the elementary corpuscles of fowlpox. This indicates that the inclusion bodies may in all cases be composed of elementary bodies. Further, the size of the virus, as estimated by the different indirect methods mentioned above, agrees with the estimated size of the elementary bodies. Further more, the elementary bodies are agglutinable by their specific serum from recovered cases and, in the case of some at least, they have been shown to be the pathogenic agents. All these indicate that elementary bodies may be the actual virus.

CHAPTER XXXVI

VIRUS DISEASES OF MAN

Rabies: Hydrophobia

Rabies is an acute infectious disease primarily of animals, particularly affecting the canine species, and occasionally transmitted to man through their bite. It is due to a virus which is present in the nervous tissues and the saliva of rabid animals. A paralytic form of rabies, transmitted by the vampire bat, has been reported from Trinidad, Brazil and other places. Some workers still hold the view that a protozoon is the cause of rabies. The term hydrophobia is sometimes confined to the disease as it occurs in man and rabies as it occurs in animals; fear of water is not a symptom in the latter.

Pseudo-rabies, or Aujeszky's disease, a virus infection of cattle, dogs and other animals, is different from rabies. By cross protection tests the virus of this disease has been shown to be antigenically distinct from the rabies virus. The former does not attack man.

The rabies virus measures about 100–150 $m\mu$ and is not readily filterable. It stimulates the production of characteristic cytoplasmic cell inclusions called Negri bodies (*vide infra*). The presence of elementary corpuscles in rabies has not been shown beyond doubt. The virus grows in the brain cells of the chick or mouse embryo. It is resistant to glycerol and probably to ether. The action of phenol is uncertain; probably it kills the virus after prolonged exposure. Desiccation attenuates the virus. The rabies virus appears to be more virulent to animals than to man.

The infection is readily transferable to dogs, rabbits, guinea-pigs, sheep and other animals. By any parenteral route it is successful. Inoculated intracerebrally, rabbits develop the disease after an incubation period of two to three weeks and die in the course of another week.

The incubation period in dogs varies from two to eight weeks. Early symptoms are behaviour changes and a perverted appetite. This is followed by a period of excitability. The animal becomes uneasy and restless and runs about aimlessly. At the slightest provocation it becomes greatly excited and furious (furious rabies). The paralytic stage then commences. There is dribbling of saliva due to inability to swallow. As paralysis deepens the animal becomes increasingly inactive and stuporous (dumb rabies) and death occurs.

The disease is an acute inflammation of the nervous system and characteristic histological changes in it are evident. The virus is found in the lesions and in the salivary glands. Hence, saliva is infectious, serving as the medium of transport of the infection. Another conspicuous feature is the occurrence of inclusion bodies in the brain.

Negri regarded these cell inclusions, which he was the first to describe in 1903, as protozoal in character and causally related to rabies. Negri bodies occur in natural as well as in experimental disease induced with the street virus. They are present in all parts of the brain but are more constant, numerous and largest in the nerve cells of the hippocampus major and the Purkinji cells of the cerebellum. They are located in the cytoplasm of the affected cells and take any shape, round, oval or angular, and vary in size from $0{\cdot}5\,\mu$–$20\,\mu$. Being acidophilic, they appear pink when stained with the Leishman, Giemsa or Mann stain. Often one or more basophilic elements can be made out within these acidophilic bodies. The exact nature of Negri bodies is still unknown, but their occurrence in rabies is specific and constant. They are absent only in the early stages of the disease. In fact, the demonstration of these bodies in the lesions of rabies has been accepted as diagnostic ever since their discovery. Some workers regarded them, as did Negri himself, as protozoal in nature. They are not developed in infections with the fixed virus.

Pasteur found that by repeated passage through rabbits the infective agent of rabies undergoes a gradual exaltation in virulence until it reaches a constant maximum with a fixed incubation period of six to seven days. This hypervirulent virus he called *fixed virus* in contradistinction to the virus from natural infection of dog or *street virus.* This modified virus has suffered a fall in its infectivity to monkeys and man, while its neurotropic

predilection has become more impressed. It has also lost the power of inciting the production of Negri bodies; may be because of the shorter incubation period.

Rabies is widely prevalent throughout the world. From a few places, like England, it has been eradicated by strict muzzling and quarantine laws. It is primarily a disease of dogs, jackals and wolves and man may get the infection through bite of these animals, usually of dogs, suffering from the disease. Transmission among animals is also through bite. The mere contamination of the skin with the infected saliva does not lead to infection as the virus cannot enter through the unbroken surface. For this to happen there must be a breach of surface.

Only about 35 per cent. of persons bitten by definitely rabid dogs develop the disease. Whether escape is due to natural immunity or to accidental causes, is not clear. The chances of rabies developing after bite depends upon several factors. A severe wound is more certain to cause infection than a superficial one. Bites on bare skin are more dangerous than bites through heavy clothing. The incubation period varies from 27–64 days, being influenced by the circumstances of bite, the nature of the wound and its proximity to the head. The spread of infection from the implantation point to the brain takes place along the nerve fibres. Hence the incubation period is long if this route is long. Conversely, the nearer the bite to the central nervous system the shorter the incubation period. Likewise, deep badly lacerated and multiple wounds ensure rapid infection and shorten the incubation period.

The virus causes an acute inflammatory condition of the central nervous system. Naked eye examination reveals no conspicuous changes. Microscopically, the affected neurons show evidence of degeneration and neuronophagia. There is also perivascular round cell infiltration. Negri bodies are present but not so regularly as in the animal lesions. The virus is present in the nervous system, but its occurrence in the cerebrospinal fluid is inconstant.

The disease is invariably fatal. Neutralising antibodies are elaborated during infection, but evidently they are inadequate to protect the patient.

Diagnosis. The laboratory diagnosis of rabies depends on the finding of Negri bodies in stained smears or sections from the

brain of the rabid dog or on the result of animal inoculation. The animal may be sick and in a few days of bite will die of characteristic clinical symptoms. If the dog is apparently healthy, it should be kept under observation for a period of fourteen days and any suggestive symptoms arising, should be killed. If it is killed too early Negri bodies would have had no time to develop. After removing the brain with aseptic care, small pieces are taken from the hippocampus situated in the floor of the inferior horn of the lateral ventricle. A piece is gently pressed and impression smears are made by applying the slide to the cut surface without the use of any pressure. Other pieces are put in formalin for preparing histological sections. Smears and sections are stained by Mann's, Leishman's or Giemsa's method and searched for the presence of Negri bodies. Further confirmation may be sought by inoculating a suspension of the brain tissue subdurally into a rabbit or mouse. Where the laboratory is at a distance, the severed head or removed brain of the dog should be sent packed in ice.

Prophylaxis. Control of rabies in dogs is the ideal preventive measure. This should be done by enforcing muzzling and quarantine laws. Once bitten, immediate and thorough cauterisation is valuable in preventing infection. It may still be useful if done within forty-eight hours after bite.

Specific prophylaxis consists in the active immunisation of the individual. The incubation period is long and if the course is started early after bite, the disease can be prevented from developing. Several factors, such as the nature and size of the wound, circumstances of exposure and the condition and availability of the dog, make it often difficult to decide whether the prophylactic treatment is indicated or not. When the wound consists only of abrasions or scratches or is superficial and remote from the head and the dog is apparently healthy, there may not be danger in withholding inoculation till the investigations are completed. Where doubt does not exist, prophylactic inoculation should be instituted immediately.

Pasteur's method was to immunise with the fixed virus attenuated by desiccation over caustic potash. Starting with the cord of infected rabbits dried for fourteen days, he worked up with cords dried for decreasing number of days thus containing virus of progressively increasing virulence, until he reached a

cord dried only for five days. He gave it as a saline suspension subcutaneously. Several modifications have been introduced in the original Pasteur's method, but the principle and technique remain the same. Fixed virus is the strain employed in all methods. Hogye's dilution method is to use the active virus diluted 1 : 4,000, start with a small dose and gradually increase it. In Babes' method inactivation of the fixed virus is accomplished by heating the cord at varying temperatures. Remlinger's method of inactivation is by etherisation.

Semple's method is to treat the virus with 1 per cent. carbolic acid. A 4 per cent. suspension of rabies material is made in sterile normal salt solution and treated with 1 per cent. phenol at 37° C. for twenty-four hours. It is then diluted with an equal volume of sterile normal saline, making a 2 per cent. suspension in 0·5 per cent. phenol. Whether the virus is killed by this process or only inactivated, is not certain. Semple's method is now followed in many places and infected sheep brain has replaced rabbit cord. A course of fourteen injections of 5 ml. each given daily by the subcutaneous route is considered sufficient.

The usefulness of antirabic vaccination is beyond doubt. It has greatly reduced the incidence of the disease. While the mortality rate among the uninoculated who develop the disease is hundred per cent., that among the vaccinated is very low, less than 1 per cent. A condition of disseminated encephalomyelitis may appear as a complication any day from the sixth to the twentieth after the commencement of the course (p. 692). But it is very uncommon and occurs only in adults.

Poliomyelitis

Acute anterior poliomyelitis, or infantile paralysis, is an acute infectious disease of viral origin, affecting the central nervous system and exhibiting a special tendency for localisation in the anterior cornua of the spinal cord. The detailed description of the disease was given by Heine (1840) and Medin (1890), hence it is often referred to as Heine-Medin disease. Our knowledge regarding its epidemiology and immunity is far from complete.

One of the smallest viruses, it measures about 12-17 $m\mu$, passing readily through bacterial filters. It is probable that this virus has a cylindrical shape, as suggested by recent studies. It stimulates the formation of inclusion bodies. It is essentially

a neurotropic virus. Heat and oxidising agents readily destroy the virus. The action of glycerol, freezing and drying is resisted for long, and when preserved at 0° C. in 50 per cent. glycerol, the virus remains viable for several years. It has been cultivated in tissue cultures, containing tissue of human embryo.

The disease can be reproduced in monkeys; the cotton rats and white mice are susceptible to certain strains of the virus. Other animals are quite refractory. Filtrates of materials from infected spinal cord and brain, when inoculated subdurally or intraperitoneally into macacus monkeys, give rise to a disease which is clinically and histologically identical to human poliomyelitis. Transmission from monkey to monkey is possible.

Acute anterior poliomyelitis affects only man. It has a world wide distribution, occurring either in epidemic or endemic form. It is more frequent in colder than in warmer climates, and in the former its highest incidence is in the late summer and early autumn. Its main incidence is in children between the ages of one and fifteen years. Infants under six months of age usually escape, possibly due to natural passive immunity acquired in utero. Curiously enough, healthy children appear to be more susceptible to the disease than the sickly. In rural areas, adults are more frequently attacked than in cities and towns. Large outbreaks occur periodically in America and in some other countries. Poliomyelitis is not so common in India.

The precise mode of transmission has not been unravelled. It is probably through droplet from cases and carriers. The portal of entry may be the nose. This theory is based on the fact that the virus can be recovered from the nasopharyngeal washings of cases and contacts. Observations on the experimental poliomyelitis in monkeys also support this view. Recent investigations show that the virus is present not only in the nasopharynx but also in the ileum and the faeces of sick persons and healthy contacts and in sewage. Limited experiments with monkeys would seem to show that the virus can parasitise and multiply on the intestinal wall. Faecal transmission through water, milk and food is, therefore, a probable method of spread. Flies also may play a role in the propagation of this infection. From the alimentary canal the virus may get into the nerves of the autonomous nervous system and pass on to the spinal cord.

How dissemination of infection takes place in the body, is not well understood. After gaining entry into the nasopharynx, the virus probably spreads *via* the olfactory nerves, olfactory lobes and finally to the anterior cornua, the selective site of activity. As the infection spreads, any portion of the brain may be attacked, but the main brunt falls on the anterior horn cells of the cervical and lumbar swellings of the cord. Acute inflammatory reaction of the affected part follows. The histological picture is one of degeneration of the affected neurons with vacuolation and neuronophagia, associated with perivascular infiltration with round cells. Intranuclear inclusions can be seen in the early stages; they are acidophilic and about 3–4 μ in size.

The incubation period varies between seven and fourteen days. In the paralytic type after a short febrile period paralytic symptoms appear. The mortality rate varies in different epidemics with an average of about 10 per cent. Permanent paralytic sequelae are frequent in the survivors. Besides the paralytic type, abortive and mild cases, not progressing to the paralytic stage, may occur. All such cases also stimulate the production of immunity. Usually, they escape detection and there is no direct proof of their occurrence, but serological investigations lend support to it. While the incidence rate of clinically recognised poliomyelitis is less than one per cent., a very much larger proportion of the adult population of endemic areas, about 70 per cent., reveal the presence of immune bodies in their blood. This is best explained on the assumption that the frequency of infection is far more than the frequency of paralytic cases and that the former leaves in its wake active immunity. It is also probable that healthy carriers exist and serve to propagate the infection.

Immunity. Solid and enduring immunity lasting for the remainder of life follows recovery from the disease and second attacks are extremely rare. It is associated with the presence of neutralising antibodies in the serum which can be easily demonstrated by neutralisation tests.

Treatment. An immune serum prepared from horses is available. Its therapeutic value as well as that of the convalescent serum has not been established. Once the virus has invaded the nerve cell, serum fails to produce any effect on it. Hence, serum should be given as early in the disease as possible and well before

the onset of paralysis. The intravenous or the intramuscular route may be chosen as the condition demands. The dose is 20–40 cubic centimetres.

Prophylaxis. Both immune and convalescent sera may also be used for prevention. A dose of 20–40 ml., administered intramuscularly, protects for about three weeks. Active immunisation against the disease with attenuated virus has been tried, with little encouraging results. There is also potential danger in it. The likely existence of immunologically distinct strains of the virus further complicates the problem. It is doubtful whether killed vaccine has any immunising value. A non-specific method has attracted some attention. It is based on the supposed efficacy of zinc sulphate in blocking the march of the virus along the nerve track. A one per cent. solution is sprayed intranasally. It is of doubtful utility.

Herpes Febrilis

Herpes febrilis, or herpes simplex, is a mild eruptive disease of the skin and mucous membranes due to a virus which is distinct from the virus of herpes zoster. The relationship of the febrilis virus with epidemic encephalitis is discussed under that disease.

The herpes virus is present in the vesicular fluid and its size is about 100–150 μ. It is resistant to 50 per cent. glycerol and low temperatures. It is cultivated in the presence of living cells and in the chorio-allantoic membrane of the chick embryo. The virus can be readily transmitted to many laboratory animals. Rabbits are particularly susceptible and the condition can be reproduced in them either by intracorneal, intradermal or intracerebral injection with filtrates of vescicular contents. Eosinophilic nuclear inclusion bodies are developed in their lesions.

Herpes febrilis is a benign acute condition, characterised by the spontaneous appearance of crops of vesicles, usually on the lips (herpes labialis), cornea or genitalia (herpes genitalis). The vesicle ruptures soon followed by encrustation and healing, leaving no scar. The virus is probably normally present in the nasopharynx of some people and is easily incited into activity by different causes, such as fevers, exposure to cold, nasal infection, emotional strain and others. Herpes is very common in pneumonia and cerebrospinal fever.

The immunity developed is very evanescent and recurrent attacks may occur at short intervals even though antibodies may be found in their blood. In contrast to this, the experimental rabbits develop marked resistance.

Herpes Zoster or Shingles

Herpes zoster is a mildly contagious and acute disease, characterised by a vesicular cutaneous eruption. It is probably due to a virus. The condition is distinct from herpes febrilis. The relationship of herpes zoster with varicella is discussed under that disease (p. 700).

Herpes virus is about 250 μ in size. It is both dermotropic and neurotropic, and besides the skin lesions, histological changes are found in the posterior root ganglia and the corresponding region of the spinal cord. The presence of intranuclear acidophile inclusion bodies in the skin lesions and of elementary bodies in the vesicular fluid has been reported. The virus is cultivable in the chorio-allantoic membrane of the developing chick embryo. Susceptible children can be successfully inoculated with filtrates of materials from vesicles, but all laboratory animals are refractory.

The characteristic rash consists of vesicles on the skin ushered in by local hyperasthesia and erythema; pain and itching are marked features. Usually, eruptions are unilateral and distributed along the course of one or more sensory nerves. One attack confers prolonged immunity.

Encephalitis

Several types of encephalitis have been described from various parts of the world. While the aetiology of some of them has been definitely proved to be a virus, the cause of others still remains obscure.

Encephalitis Lethargica. This is an infectious disease of unknown aetiology. All attempts to discover the cause have proved futile. The great stumbling block in the way is the absence of susceptible animals. A virus hypothesis has been put forward. Some workers claim to have isolated the virus of herpes febrilis from cases of encephalitis lethargica and reproduced an identical condition in monkeys and rabbits by inoculating with the same virus intracerebrally. Others have failed to corroborate this and the claim for the herpes virus remains unproved.

The disease occurs in periodic waves and recent years have witnessed great decline in its incidence. This latter observation is probably not altogether correct, and mild cases and inapparent infection may be occurring, as judged from the fact that the sequelae of the disease are by no means very rare. At present it is endemic in all parts of the world. The disease occurs in the epidemic as well as in the sporadic form. The central nervous system is the site of activity of the infective agent, the main brunt falling on the grey matter. The mid brain, basal ganglia, pons and medulla suffer most. Characteristic histological changes, consisting of perivascular and diffuse infiltration side by side with degeneration of the neurons, are present in the grey matter. The mortality rate is 30–40 per cent. Serious sequelae may be left on the wake of the disease.

St. Louis Encephalitis. This type was first recognised during an epidemic at St. Louis in the U.S.A. It is probably present in many parts of the United States. Its virus aetiology was proved by the isolation of a virus from the brain of patients after death. It is cultivable in tissue culture and in the yolk-sac of the fertilised hen's egg. There is some minor antigenic relationship between this virus, West Nile virus and the virus of type B encephalitis of Japan. The condition can be reproduced in monkeys and mice by the intracerebral inoculation of the virus. The mosquito appears to be the transmitting agent of infection. There is no complete proof to incriminate any animal as the natural reservoir of infection; probably, many birds and mammals are naturally infected.

Australian X Disease. This is another form of encephalitis of proved viral origin. Close relationship has been shown to exist between the virus of this disease and that of louping-ill, a virus disease of sheep. The former is also related to the virus of type B encephalitis of Japan. The disease has been reproduced in monkeys and mice.

Japanese Encephalitis. Two types of encephalitis, type A and type B, occur in Japan. The former is probably identical with encephalitis lethargica. Type B has been proved to be due to a virus, the size of which has been estimated to be about 20–30$m\mu$. It can be grown by the tissue culture and the chick embryo methods. The disease is communicable to rabbits, guinea-pigs, mice and monkeys. The mosquito may be the transmitting vector; many

culicine species can be experimentally infected and the virus persists in the mosquito for several weeks.

West Nile Encephalitis. The virus of this form of encephalitis is antigenically related to the virus of type B encephalitis of Japan and to the St. Louis virus. It is infective for mice and rhesus monkeys.

Equine Encephalomyelitis. This is a natural virus disease of horses first reported from North America. Two types have been described, the " western " and the " eastern ". The occurrence of both types in man has been reported.

Acute Disseminated Encephalomyelitis. An acute form of disseminated encephalomyelitis, involving the whole of the central nervous system, may rarely occur as a post-vaccinal sequela, after certain infectious diseases like smallpox, varicella, measles, mumps and influenza, and during antirabic inoculation. The clinical and histological pictures are similar whatever be the cause. The condition is distinct from encephalitis lethargica and acute poliomyelitis. In contrast to the neurological changes produced by the known neurotropic viruses, perivascular demyelination is the most conspicuous feature of this condition.

The post-vaccinal form occurs about 7–14 days after vaccination. Young infants are immune. It is more frequent after late primary vaccination; though seldom, it has also occurred after revaccination. The onset is sudden, and the course lasts for about a week. The death rate is about 50–60 per cent. Recovery may take place even after the onset of paralysis and it may be complete.

Histologically, demyelination, perivascular infiltration and large areas of perivascular softening are conspicuous features. Though the lesion is diffuse, the white matter of the cerebrum, brain stem, pons and medulla are more severely affected. The cord also may be involved, especially of the lumbar region. The perivascular demyelination and the diffuse nature of infiltration, spreading from the perivascular areas and diminishing to the periphery, mark out this condition from encephalitis, poliomyelitis and rabies.

So far the search for a cause has proved unsuccessful. The condition has been variously suggested to be due to the virus of the primary disease itself, to some other latent virus activated by vaccination or the primary infection, to a fresh viral infection, to some toxic products and to allergy.

Acute Lymphocytic Chorio-Meningitis

This is a benign form of meningitis due to a filterable virus and often running a prolonged course. It is also referred to as acute aseptic meningitis.

The virus is 37–60 *mμ* in diameter. It grows on the yolk-sac of hen's egg. The condition can be transmitted to mice, monkeys and guinea-pigs by the intracerebral injection of the cerebrospinal fluid obtained from the patient. But it should be remembered that the infection occurs naturally in mice. The source of human infection and the mode of transport are both obscure.

In the early stage of infection the virus is present in the blood; later as meningeal symptoms appear the organism is present in the cerebrospinal fluid. Eosinophilic nuclear inclusion bodies have been described in the brain. Both neutralising and complement-fixing antibodies are present in the serum of human cases, contacts and of experimental animals. The cerebrospinal fluid is sterile for ordinary bacteria. It shows a lymphocytic increase of 200 or more cells per cubic centimetre. Complete recovery is the rule. The diagnosis can be established by the intracerebral inoculation of 0·02 c.c. of spinal fluid into a healthy mouse which takes ill in about six days and dies in another two or three days.

Variola or Smallpox

Smallpox is a highly contagious exanthematous disease caused by a virus. Alastrim is now accepted as a mild form of smallpox due to an attenuated strain of the same virus. No immunological distinction can be made out between the virus of alastrim and the variola virus.

The size of the variola virus is 125–175 *mμ*. It does not readily pass through bacterial filters. It is present in the vesicular fluid and the encrustation of the specific lesions. Cell inclusions, *Guarnieri bodies*, are formed in the lesions and elementary or *Paschen bodies* are demonstrable in the vesicular contents. The virus has been cultivated in the rabbit's testicle, tissue culture and in the developing chick embryo.

The smallpox virus is not very virulent to the laboratory animals; inoculated intradermally into the calf or rabbit, it produces only a mild local lesion. Monkeys are more reactive and

develop on intracutaneous inoculation a typical eruption which goes through the usual evolutionary stages seen in smallpox. By successive passage through these animals, the virus undergoes certain biological changes, acquiring eventually all the characteristics of the vaccinia virus. This transformed vaccinia virus has become attenuated and lost its ability to cause variola and on reinoculation into man produces only vaccinia. In all these respects, the virus of alastrim behaves in the same way as the virus of variola.

Smallpox is widely distributed in the tropics and subtropics and in these regions it is still a devastating disease. At one time it was equally prevalent and serious in the temperate regions too, but, as the result of extensive prophylactic immunisation and improved public health measures, it is now relatively infrequent and milder in those places. America has the second heaviest incidence with India leading. But in the former country it is the mild alastrim type that is prevalent. Natural immunity to smallpox either does not exist or is very rare. Variola is one of the most contagious of all infectious diseases, and race, age, sex and similar factors do not influence its incidence. Usually smallpox occurs in the form of epidemics of varying extent and severity. Sporadic cases also occur. A reduction in the severity of the disease and presumably in the virulence of the organism during the last fifty years has been reported. It may be only apparent and explainable by the progressive increase in the practice of protective vaccination. Inapparent infection and silent immunisation may also be occurring, stepping up the mass immunity.

Transmission. The precise mode of transmission is unknown. The patient is infectious not only throughout the clinical course but also during the incubation period, thus rendering quarantine an inadequate protective measure. The body after death is still a source of infection. The virus is present in the skin lesions and probably in the nasopharyngeal secretions. Direct transmission through infected droplets and other air-borne particles appear to be the method. They gain entry through the upper respiratory tract and the virus primarily settles in the nasopharynx. Since the virus resists desiccation, transfer through dust containing dry scales and minute particles of crust cannot be ruled out. Direct implantation of variolous material into the skin does not seem to play any important part in transmission.

A pregnant woman contracting infection may transfer it to her progeny. Contact carriers may also serve to spread the infection for a limited period.

From the nasopharynx the virus is distributed throughout the body, reaching the skin through the blood stream. It can be demonstrated in the blood and internal organs.

The incubation period varies from 9–15 days. The disease starts with high fever, severe headache and other general symptoms. The specific rash appears on the third day in the form of discrete shotty papules. The evolution of the rash proceeds from papule through vesicle and pustule to encrustation and scarring or pitting. Pustulation is associated with secondary infection with pyogenic cocci. Variola may rarely occur without the rash; it is also infectious. Inclusion bodies, first described by Guarnieri (1892), are present in the cytoplasm of the epithelial cells of the deeper layers of the pock. Elementary or Paschen bodies are likewise present in the lesions, which can be demonstrated in the papular and vesicular stages of the disease. Considerable disfigurement may be left on the wake of the disease. The mortality rate is high, 30 per cent. or over in severe epidemics.

Immunity. Survival from smallpox is generally accompanied by a high degree of resistance, lasting for the balance of life. But second and even third attacks may occur; they are very mild. The serum of the immune person contains antibodies: neutralising, complement-fixing, flocculating and agglutinating. The neutralising antibody is probably virucidal, and its presence can be shown by neutralisation tests in suitable animals; the presence of others are revealed by appropriate in vitro tests using as antigen materials from vesicles or cowpox.

Vaccinia, or cowpox, is due to a filterable virus. It is disease confined to the udder and teats. The virus is 125–175 $m\mu$ in size and is not readily filterable. It is easily communicable to calves and rabbits. From cows man may get vaccinia, but, as in cows, only localised lesions are produced at the inoculation points and without causing general disturbance. As a result, he develops immunity not only against vaccinia but also against variola. It is this observation of Jenner that paved the way for developing protective vaccination against smallpox on rational lines (p. 225).

No decision has yet been reached about the relationship of the cowpox virus with the variola virus. Slight antigenic differ-

ence between the former and the vaccinia virus modified from the smallpox virus has been reported. But the term vaccinia is used for both regardless of the origin. A number of pock diseases due to virus occur naturally in man and animals; smallpox and alastrim, cowpox, sheeppox, swinepox, fowlpox and others. Close resemblance exists among these, but whether these diseases are due to the same organism or whether there is a specific one for each, is not clear. These viruses may be closely related. It is possible that they have a common ancestor in the cowpox virus and that the varying characteristics are the result of adaptation through long sojourn in different host species. Another view is that the variola virus is the parent strain from which all others have sprung. Possibly, all these viruses can be converted into vaccinia virus through suitable animal passage and, notwithstanding any minor serological differences between such biological variants, effective cross protection among them may be possible. This has been fully established in the case of the viruses of smallpox and cowpox.

Several strains of vaccinia virus are now in use for the preparation of vaccine lymph. Some of them are originally derived from cowpox, while others are probably modified variola strains. They are readily transferable into calves and rabbits and are maintained with full potency by alternate inoculation into these animals. A good calf lymph will cause a lesion in a minimum dilution of 1 : 1,000. Inoculated into scarified skin of the calf or rabbit, it produces swelling and erythema going on to a definite papule by the third day, vesicle containing clear fluid by the fifth day and pustulation and scabbing in 8–10 days after inoculation. Histologically, epithelial cells of the lesions show large intracytoplasmic Guarnieri bodies; elementary or Paschen bodies are also present in the lesions. If the calf lymph is inoculated intraperitoneally into the rabbit in suitable doses, generalised eruption on the skin and lesions in the internal organs occur. Serial passage through the rabbit's brain gives rise to a fixed virus called *neurovaccine*. In some of the European countries this is used for vaccination instead of calf lymph.

The vaccinia virus can be cultivated in minced fresh kidney from hen or rabbit suspended in Tyrode's solution or in minced chick embryo suspended in the same fluid or in the chorio-allantoic membrane of the developing chick. Elementary bodies are demonstrable in such cultures.

Vaccine Lymph. Vaccine prepared for prophylactic immunisation against smallpox is called *vaccine lymph* or *calf lymph.* It consists of vaccinia virus grown usually on cows together with the products of inflammation, preserved in 50 per cent. glycerol in order to destroy the extraneous organisms, like staphylococci and streptococci. As glycerol is not lethal to the virus, the active agent in immunisation is the live virus.

Healthy female calves, 4–6 months old, are chosen. Scarifications or linear incisions are made on their abdomen after depilation and thorough cleansing. The seed lymph, which is kept fully active by frequent passage through rabbits, is then inseminated into the cut surface. Rigorous precautions about cleanliness should be taken. By the fifth day pustulation would have ensued when the lesions are scraped with a spoon with aseptic care and the material (pulp) is emulsified with 50 per cent. glycerol. The mixture is preserved in the cold in sealed containers for several weeks; by this the bacterial content of it would have considerably diminished. Sterility tests, both aerobic and anaerobic, are conducted periodically. Complete bacteriological purity is hard to achieve. After a final testing for sterility and potency the product is sent out in vials or capillary tubes for vaccination. If properly preserved in the dark and cold, the vaccine remains unimpaired for three months.

Diagnosis. The diagnosis of smallpox is easy and no laboratory tests are usually required. Rarely some difficulty may be encountered in differentiating it from chickenpox. In doubtful cases a flocculation test may be done by mixing the ground up materials from pocks with antivaccinal rabbit serum and incubating the mixture at 37° C. A positive reaction is obtained in variola but not in varicella. A positive agglutination reaction is likewise obtained in smallpox with the patient's serum and a suspension of Paschen bodies. For testing the presence of complement-fixing antibody in the patient's serum, a saline suspension of variolous crusts may be used as antigen. Paul's test consists in the inoculation of the vesicular or pustular contents into the scarified cornea of a rabbit. In positive cases, a keratitis develops in which Guarnieri bodies are demonstrable. A negative reaction, as it does not exclude variola, has no value. Similar lesions are not produced with varicella pus.

Prophylaxis. General measures as in any other acute infectious disease are indicated. Segregation of the sick and quarantine of the exposed for a period of fourteen days are useful.

Specific prophylaxis is by vaccination. While no specific therapeutic measures are available against smallpox, effective and lasting protection can be induced in the susceptible subject by active immunisation with living vaccinia virus. Active immunisation had been practised with smallpox pus from very ancient times in China and India, but our present knowledge about it dates from 1796 when Edward Jenner made the epoch-making observation that milkmaids who had suffered from cowpox were immune to smallpox. During recent times considerable advances have been made in the theoretical aspect of vaccination and certain improvements, such as the use of glycerine to control the contaminating bacteria, have been made on the practical side. The preparation of smallpox vaccine has been described above. Calf lymph is the one now generally used. Lymph from vaccinal lesions from other animals has also been found satisfactory. Variola virus from infected patients is too risky. Neurovaccine (p. 696) is used in certain places in Europe. In America limited trials have been conducted with the culture virus. It has the advantage that it is bacteria-free.

Vaccination is performed by cutaneous scarification. The multiple puncture method appears to be superior to other methods. After gentle but thorough cleansing, the lymph is deposited on the skin and light scarification made through it. A local lesion is produced which shows all the sequence of events seen in smallpox. But it is not contagious though autoinoculation may occur. In primary vaccination, the height of reaction is reached in nine to ten days after inoculation, but this is shortened in subsequent vaccinations. The reaction is essentially a local manifestation, but there is also general diffusion of infection throughout the body, engendering active immunity of a high grade. In those who had been previously immunised, either as the result of natural infection or vaccination, the intensity of reaction varies and reaches its zenith after a shorter interval than in primary vaccination. In a person possessing a high degree of immunity, the maximum reaction is a papule appearing within three days after inoculation. This type of reaction is called the *immune reaction*. Between this and the type of primary reaction, intermediate types occur

indicative of grades of partial immunity, *accelerated* or *vaccinoid reaction*. A primary type of reaction signifies a complete breakdown, and absence, of immunity. Absence of reaction after primary vaccination means failure of vaccination due to impotent lymph or faulty technique.

The best period for primary vaccination is between six weeks and six months, done preferably during the cold weather. Immunity is found to develop in about eight days after successful inoculation. Hence, vaccination immediately after exposure prevents or modifies the disease. The area of foveation, and not its number, is considered to be a rough indication of the strength of immunity. The immunity acquired is of a high degree and lasts for a minimum of five to seven years; it may be longer but usually not life-long. Hence, vaccination should be repeated before the seventh year and again whenever there is the prospect of exposure. It is a safe practice to revaccinate whenever there is an outbreak of smallpox epidemic, and this applies even to those who have had the disease. As a community constantly receives unprotected newcomers, all such susceptibles should be regularly vaccinated. Also, whenever there is a threat or actual outbreak of epidemic everybody should be revaccinated regardless of recent vaccination or disease.

Some of the rare complications of vaccination are suppuration of the lesion due to the presence of pyogenic cocci in the lymph or to secondary infection, generalised vaccinia, tetanus and encephalomyelitis (p. 692). The last named is a dangerous complication mostly following primary vaccination in older children. It seldom occurs after revaccination. A number of cases occurred at one time, but now it is extremely rare. Its cause is unknown. The vaccine lymph does not seem to be the direct cause. Early primary vaccination has been advocated as a preventive.

Undoubtedly, vaccination is of great value in the prevention of smallpox; in fact, it is the only one of any value. It has reduced not only the incidence of smallpox but also its morbidity and mortality. Statistical evidence is in full harmony with this conclusion.

Chickenpox or Varicella

Chickenpox, or varicella, is an acute and highly contagious disease, characterised by a vesicular eruption occurring in crops. Its cause is most likely a virus.

It has a close aetiological relationship with herpes zoster. The viruses of both are either identical or closely related as indicated by the striking cross immunity between them. The serum of varicella patients gives positive complement-fixation reaction with antigen derived not only from varicella but also from herpes zoster. Intranuclear acidophile cell inclusions, similar to those found in herpes zoster, are found in the epithelial and endothelial cells of the varicella lesions. Elementary bodies have likewise been demonstrated in the vesicle fluid of both. Further, with the vesicular contents of herpes zoster, it has been possible to produce varicella in susceptible children but not in those who have had chickenpox. Such successful inoculation renders them immune to varicella. Clinically, the non-immune contacts of herpes zoster may develop typical chickenpox and contacts of chickenpox may develop zoster. A point of difference is that while the virus of chickenpox is extremely infectious, that of zoster evinces only a low order of infectivity.

Varicella is readily communicable to children by the inoculation of filtrates of vesicular material. By the intratesticular inoculation with the same material, it is also transferable to vervet monkeys, in which cell inclusions are also developed.

Varicella is more frequent in children. Usually far milder in nature than smallpox, it has a general epidemiological and clinical resemblance to it, but the two are not causally related. As in the case of smallpox, transmission is probably through the nasopharyngeal route. The incubation period is two to three weeks. The disease is most infectious in the early stages, and after about ten days from the appearance of the first crop of eruption, it is no more infectious.

An attack of chickenpox usually confers life-long protection. Second attacks may occur. Neutralising antibodies are present in the blood; agglutinins, precipitins and complement-fixing antibodies may also occur.

Active immunisation with the vesicular fluid is possible but seldom practised. Convalescent serum has protective properties and may be used both for prophylaxis and treatment.

Measles

Measles is an acute highly infectious febrile disease usually occurring in epidemic form and primarily affecting children.

Very little is known about its aetiology. It has not been possible to find out a susceptible animal. The suggestion, based on the results of human and animal experiments, that a virus is its cause is highly probable; it is further supported by the consistent failure to isolate any organism from measles and also by the nature of the immunity engendered in the disease.

Some workers claim to have reproduced the disease in monkeys and man by inoculating them with blood taken from measles cases at the height of fever. From these inoculated cases they have isolated a virus. Further, they have been able to pass the infection serially through these animals.

Filtrates of blood and nasopharyngeal washings from cases of measles give rise to inapparent growth in the chick embryo membrane and from this successive subcultures can be obtained in fresh eggs. With this culture material the disease has been reproduced in monkeys.

It has been claimed that by intratesticular inoculation the infective agent can be maintained in the rabbit and that after a number of passages in these animals the virus can produce the typical disease on reinoculation into man.

Measles is one of the commonest acute infectious diseases and natural immunity to it does not exist in man. Usually the disease occurs in epidemic form. It is more serious in the higher latitudes. Measles is primarily a disease of childhood, about 80–90 per cent. of cases occurring below the age of eight years. Young infants below five to six months generally do not get it, presumably because of passive immunity inherited from mother.

Most likely the infection is transmitted by droplet and enters through the upper respiratory tract. The possibility of intra-uterine infection has been suggested. Measles is not infectious during the incubation period; it is highly infectious during its clinical course and ceases to be communicable after the rash has faded.

The incubation period of measles is about 10–11 days. The early appearance of Koplik spots is characteristic. Uncomplicated cases run a definite course and show a mortality rate of less than 1–2 per cent.

The disease is dangerous because of the serious complications and sequelae it is liable to. The infection renders the mucous membrane of the upper respiratory tract very unhealthy, paving

the way for secondary bacterial invaders. The latter may cause fatal bronchopneumonia or other serious complications, like otitis media, mastoiditis, sinusitis and encephalitis. Again, measles not only favours infection with the tubercle bacillus but also activates a latent tuberculosis.

Immunity. The immunity acquired as the result of an attack of measles is solid and life-long; second attacks are exceedingly rare. Convalescent serum has marked protective properties. Antibodies tend to persist in the blood throughout life, albeit in diminished concentration. They are also found in the placenta and an extract of it, called *immune globulin*, is found to have protective properties. Adult serum also possesses protective powers, either due to measles in childhood or to subclinical infection.

A peculiar feature of measles is that it temporarily abolishes or weakens bacterial immunity and allergy. A prior positive tuberculin reaction may become negative or weak during the course of the disease and for some time after. So also an existing asthma or eczema may wane or disappear temporarily. Similarly, a fall in the titre of typhoid agglutinins has been noted.

Prophylaxis. No method of active immunisation is available. Passive immunisation is an effective means of preventing measles temporarily or attenuating it after exposure. It is done by administering convalescent serum intramuscularly. The dose is 10 ml. for children under the age of three years and double that amount for those over three years. The passive immunity so conferred wears out in about three weeks. Whether complete protection or attenuation is to follow the injection, is determined largely by the time of injection after exposure. If it is given within four days after exposure, about 95 per cent. escape disease from that exposure; a smaller dose given during this period instead of aborting the infection will modify it, enabling the patient to develop active immunity without the risk of a serious disease. If the serum is given between the fifth and ninth day, the incubation period is prolonged, the disease rendered mild and complications and sequelae avoided or reduced in severity, while active immunity is freely developed. But, if it is delayed beyond the ninth day after exposure, no appreciable effect on the course of the disease and on the incidence of complications is noted.

Immune globulin (placental extract) or adult serum may be used for prophylaxis in the absence of convalescent serum. The

first one is more potent than adult serum, and convalescent serum is more powerful than either. The dose of immune serum is 2 ml. for children under three years of age. The dose of adult serum for the same age period is 20 ml.; double the quantity of citrated blood may replace serum. Placental extract may cause local and general reactions but not sera. Recently, gamma globulins have been reported upon very favourably from America.

As attenuation of the disease does not interfere with the development of active immunity, attenuation is preferable to prevention in the case of healthy children. But in the case of debilitated or very young children prevention is what is sought for.

Serum Therapy. Convalescent serum may have some value if given in large doses, 40–50 ml. intravenously, before the appearance of eruption. Immune globulin has also been used.

German Measles

German measles is a milder disease than measles. Its aetiology is unknown. One attack confers lasting protection. Although there is clinical similarity between German measles and measles, the former is aetiologically distinct from the latter as shown by their immunological differences.

Common Cold

Common cold, or acute coryza, is an acute infectious disease affecting the mucous membrane of the upper respiratory tract. A filterable virus has been incriminated as its cause.

Experimental transmission of the disease to man and chimpanzees is possible; other animals are refractory. The virus increases in virulence by transmission in series through man. It is cultivable in minced chick embryo suspended in Tyrode's solution; concentrated filtrates of nasal washings from cases of cold form the inoculum. The disease can be reproduced in man by the intranasal instillation of the culture virus obtained after several subcultures in this medium.

Cold is a very widely prevalent condition affecting the nasopharyngeal mucosa. Transmission is direct through droplet. The incubation period is short, 12–48 hours. Certain factors predispose to attack: sudden chilling after exercise, sudden entry into close and stuffy rooms, exposure to drafts, wet feet and

others. The condition is trivial but very troublesome. It may be followed by secondary bacterial infection which may prolong the cold and also lead to complications like chronic rhinitis, sinusitis and bronchitis. The staphylococcus is a very common secondary invader; the streptococcus and pneumococcus are others.

Man has no natural resistance to common cold; on the other hand, ready susceptibility is the striking experience. Immunity is not developed from an attack or, if developed, is very transient.

No specific prophylaxis or therapy against common cold is available. Immunisation against the secondary bacterial invaders with autogenous vaccine is useful and will limit the course of cold to its brief duration.

Influenza

Influenza is an acute febrile disease caused by a virus. *H. influenzae*, regarded as the cause of influenza for more than three decades after its discovery in 1892, is not the primary cause of influenza, though it may play a frequent and important role as a secondary invader. The virus of influenza was discovered in 1933 by Smith, Andrews and Laidlaw. Their work has since been confirmed by the recovery of the same or but slightly different viruses from outbreaks of influenza in different parts of the world. The virus has not been isolated from endemic cases. Such cases are difficult to diagnose with certainty and the failure may have been due to a faulty diagnosis of influenza; it may be remembered that influenza very often does not conform to any definite clinical pattern.

The virus is about 80–120 $m\mu$ in diameter. Recent estimates would put it as 70–100 $m\mu$. The virus grows in tissue cultures, in the chorio-allantoic membrane of the chick embryo and in the allantoic fluid. Virus emulsions from these are used as antigens. While the virus particles are involved in the neutralisation reaction, a soluble antigen present in the emulsion and apparently formed by the growing virus would seem to be the antigen responsible for the complement-fixation test.

Recent work indicates that there are immunologically different strains of the influenza virus. Two strains, designated A and B, are definitely known. The neutralising antibodies developed against one are specific to it and do not neutralise the other.

Unlike the original A virus, the B strain, first isolated in America, cannot be easily communicated to ferrets and mice. Influenza A is considered to be the more frequent cause of epidemics, but in America B appears to be commoner. The strains of influenza A do not appear to be antigenically homogeneous; cross immunity tests reveal the existence of minor differences among them; so also with influenza B.

The influenza virus shows a peculiar faculty of agglutinating the erythrocytes of the chick. The agglutinating power is inhibited by serum containing neutralising antibodies against the influenza virus. Further, the inhibiting action of such antisera is specific with regards to the different strains of the virus, either A or B. That is, the antiserum against influenza A will exert its inhibiting action only on virus A and not on virus B and *vice versa*. Hence, it may be employed in the identification of strains.

Ferrets are susceptible and the disease can be reproduced in them by the intranasal instillation of filtrates of nasal washings from influenza patients. After an incubation period of 48 hours, a characteristic disease develops in them. It lasts for a few days and never ends fatally. The introduction of the material by the paranasal routes does not succeed, though it incites the production of immune bodies. After recovery, the experimental ferret is resistant to further infection for a period of about three months and protective antibodies are present in its blood. Ferret to ferret transmission may occur through contact or can be easily accomplished by the intranasal instillation with the sick animal's bacteria-free nasal washings. Only in the nasal mucous membrane, and not in any other situation, can the virus be detected in the infected ferret. Direct man to mouse transmission is not possible; but the ferret-adapted virus can be transmitted to mice causing a similar disease, associated also with bronchopneumonia of which the animal usually dies in three to five days. It is also possible to communicate the infection to hedgehogs.

Influenza is of universal distribution. It occurs in pandemic, epidemic and endemic or sporadic forms. Climate, season, race and other factors have no influence on its incidence. Periodic fluctuations in the severity of infection is a conspicuous feature. During the pandemic of 1918, more than 500 million people were attacked with a fatality estimated variously as 15–25 million. The mode of infection is through droplet entering through the

upper respiratory tract. The virus is found in the nasopharynx of the sick person. The incubation period is one to three days. The clinical picture presents a variety of forms, not sticking to any one particular type. The disease is dreaded not so much for the severity of the symptoms as for the after-effects, grave complications and sequelae.

An identical disease occurs in the swine. The virus causing it is not the same as that of human influenza, but they share a common antigenic factor. The former also causes an identical condition in ferrets and mice as the latter, suggesting antigenic relationship. There is also a bacillus, *H. suis*, found in association with swine influenza. While in human influenza Pfeiffer's bacillus is only a secondary invader, swine influenza appears to be a conjoint infection by the bacillus and the virus.

Immunity. Man does not possess natural immunity against influenza. The presence of a natural virus-inactivating agent in the human nasal secretions has been recently demonstrated (p. 165). It is active not only against all strains of the influenza virus but also against a few other viruses like those of herpes and louping-ill. The viruses of vaccinia, ectromelia and psittacosis are not susceptible to its action. Influenza is one of those diseases which develop only a transient acquired immunity. Recurrent attacks in the same individual are common; but there is the possibility that such attacks are due to serologically different strains. In the recovered patient's serum, neutralising, complement-fixing and precipitating antibodies are developed and can be demonstrated by appropriate methods. The complement-fixing antigen appears to be common to the several serological strains of the human influenza virus as well as to the virus of swine influenza. Curiously enough, the presence of antibodies is not a sufficient guarantee against fresh attacks. A fair percentage of normal adults show such antibodies in their blood. This is probably an indication that subclinical and unrecognised infections commonly occur. Recovered ferrets likewise contain these antibodies in the blood.

Treatment. An anti-unfluenzal serum has been produced by immunising horses. It contains a high content of antibodies and confers some protection to ferrets and mice. Neither this immune serum nor the convalescent serum is of any appreciable value in treatment or prevention.

Prophylaxis. Attempts to induce active immunity in man has so far yielded only inconclusive results. A formalinised mouse lung virus and the virus grown in chick embryo have both been tried. They confer some protection in ferrets and mice. The multiplicity of serological strains may partially explain the failure in man.

Yellow Fever

Yellow fever is an acute highly infectious disease transmitted by mosquitoes. The American Yellow Fever Commission showed in 1900 that yellow fever was due to a filterable virus and transmitted by mosquitoes. This finding was confirmed in 1928 by Stokes, Bauer and Hudson, finally disproving Noguchi's claim that a leptospira was the cause. Further, they discovered that the disease could be communicated to the rhesus monkey. Later, it was proved that the jungle yellow fever, primarily affecting animals in the rural and jungle areas and occasionally transmitted to man, was also due to the same virus. There is now increasing evidence to show that yellow fever is primarily an enzootic.

The yellow fever virus is one of the smallest viruses and is about 18–28 *mμ* in size. Nuclear inclusions are found in yellow fever. Cold, drying and 50 per cent. glycerine are not lethal to the virus. Growth occurs in the chorio-allantoic membrane of the fertilised hen's egg and in the chick embryo medium.

The infection can be readily communicated to rhesus monkeys either through the bites of infected mosquitoes or by the inoculation of infected material taken from viscera, usually the liver, after death. The virus is present in high concentration in the blood of infected monkeys. Inoculated subdurally, the viscerotropic virus causes encephalitis in mice. By serial passage through their brain it acquires marked neurotropic properties, losing its virulence for man and monkeys. The neurotropic variant, however, can still immunise against the viscerotropic strains. The brain-fixed virus produces encephalitis in monkeys, guinea-pigs and a few other rodents.

Transmission. Transmission by the mosquito, *Aedes aegypti*, originally suggested by Findlay, was proved by the American Yellow Fever Commission. But this seems to be only part of the picture. In its endemic areas, Africa and South America, yellow

fever appears to be primarily a disease of certain animals, particularly monkeys, found in the locality. In them it exists as an inapparent infection, transmitted among them by forest mosquitoes. Thus, monkeys appear to play the role of the initial reservoir host. From them *Aedes* may be getting the infection in the first instance and then spreading it among man, when he also serves as the reservoir of infection for the mosquitoes. Besides *A. aegypti*, other related species have also been found to be carriers among man. Man is infectious during the first 3 or 4 days of the disease and from him the mosquito gets the infection through bite during this period. For twelve days after the infectious feed the mosquito is not infective. This period is called the extrinsic incubation period of yellow fever. What ahppens to the virus during this period, is not clear; probably it is multiplying. The infected mosquito remains so for the rest of its life. The virus may also enter through the unbroken skin; through this channel infection among laboratory workers has been reported.

Yellow fever is confined to the tropical and subtropical regions of West Africa, South America and the islands lying between them. There is evidence to show that certain other parts of Africa are also affected. All persons are susceptible and nothing except a previously acquired immunity can protect against yellow fever. The disease is relatively benign in children and most of the fatalities are in older subjects. Unrecognised infections may also be occurring in the former. The incubation period in man is about 3–5 days or longer. During the first 3 or 4 days after the onset, and probably also during the late incubation period, the virus is present in the blood and the internal organs, particularly the liver, in which are found characteristic acidophile nuclear inclusions. Jaundice and haemorrhage are prominent post-mortem appearances. The liver is found to be the seat of greatest damage, and the virus appears to pick it out more than any other organ for inflicting its ravages. Acute and extensive fatty and necrotic changes (Councilman lesions) are present in the liver (mid-zonal) and, to a less degree, kidney. The mortality rate varies between 20 to 90 per cent.

Immunity. Natural immunity to yellow fever probably does not exist. Acquired immunity from disease is solid and life-long, second attacks being extremely rare. It is associated with the presence of virucidal antibodies in the blood which can be

demonstrated by the neutralisation test in mice—the mouse protection test. This test is extensively employed for mapping out the endemic prevalence of infection. The virus and the serum under investigation are injected intraperitoneally into the mouse following an injection of starch solution into the brain. If the serum is negative for immune bodies, the animal develops encephalitis. By the mouse protection test the disease has been shown to be widely prevalent in the endemic regions. A method of rapid diagnosis after death has been developed; it is by the histological examination of tissues removed from the liver by means of the viscerotome. Where the disease is prevalent a certain degree of endemic immunity is established, and the sera of adults contain protective antibodies; mild and inapparent infections occurring in childhood may account for this. Sera of monkeys in the endemic regions are also found positive for antiviral antibodies. The explanation of this is not quite clear (*vide supra*).

Prophylaxis. Long before the aetiology of yellow fever was established, mosquito transmission had been suggested and mosquito control as a preventive measure had been advocated. Extensive anti-mosquito measures in certain endemic areas have eradicated the disease from those areas, proving the great importance of such measures in prevention. The introduction of yellow fever to a hitherto uninfected country should be vigilantly guarded against. Modern expansion of air transport has increased the danger of dissemination. It is a great threat to countries now free from yellow fever. Infected mosquitoes as well as individuals incubating the disease may be easily transported from Africa to this country. If it happens, the infection is bound to spread with great rapidity and virulence because of the large mosquito population and of the total absence of immunity in people. Such contingencies should be met by adequate transport and quarantine laws, rigidly enforced.

Active immunisation is very valuable in prevention. Several types of vaccines have been tried. A mixture containing immune serum and the mouse-fixed virus was found to be potent, but it had to be given up due to the serious serum reactions it engendered. An attenuated pantropic virus, grown in chick embryo medium, has been found to be highly immunising and less productive of reactions. The dose is 0·5–1 ml. given subcutaneously once.

Immunity develops in about fifteen days after injection and lasts for a minimum of four years.

Psittacosis

Psittacosis is an acute infectious disease primarily of parrots but occasionally transmitted to man. giving rise to symptoms simulating typhoid fever with pulmonary complications. Besides parrots, it occurs naturally in a few more species of birds. The cause is a filterable virus; the old belief that a salmonella described by Nocard, which turned out to be *Bact. typhi-murium*, is the cause has been discarded. Pacheco's disease of parrots is different from psittacosis and does not infect man.

The psittacosis virus is one of the largest viruses, measuring 200–300 $m\mu$, and is held back by the fine bacterial filters. Unlike the vegetative bacteria, it withstands the action of glycerine but not as well as other viruses. Elementary bodies are found in the lesions as well as in the culture. Recent work tends to show that there is probably some relationship among the viruses of psittacosis, lymphogranuloma inguinale, trachoma and inclusion conjunctivitis; positive cross immunity tests have been reported among them (p. 712). Like other viruses, the psittacosis virus cannot be cultivated in the absence of living cells. Growth occurs in tissue culture and in the developing chick. Many developmental phases have been described as occurring during growth.

Elementary bodies are present in the infected tissues and, when stained by the Giemsa or Castaneda's method, can be seen under the ordinary microscope as round or slightly oval bodies, arranged singly or in pairs. They occur in large numbers in the reticulo-endothelial cells as well as free. These bodies can be obtained free of all extraneous matter when they are found to function as efficient specific antigens in agglutination and complement-fixation reactions, indicating their virus identity.

The disease can be reproduced in parrots and many other species of birds, guinea-pigs, mice and monkeys. In both experimental and natural infections, the virus is found in the blood and internal organs.

Psittacosis is a very rare disease. It is more common in some of the South American Countries. Though the association of human psittacosis with the parrot disease was long known, its exact mode of infection is still obscure. Probably, it is through the

nasopharyngeal route. Transmission from man to man is very uncommon. Its infectivity is very high and so indirect transmission through fomites is also likely. Pneumonic affection of the lung is a prominent feature in man. The disease is fairly severe with a mortality rate of 20–30 per cent. The virus appears in the sputum in two or three days after the onset of the disease and persists in it throughout the course. It is also found in the blood and internal organs, particularly the lungs, liver and spleen.

Immunity. Recovery from an attack is followed by a high grade and enduring immunity. Second attacks may not occur. Complement-fixing antibodies are present in the serum of recovered cases. The complement-fixation reaction with the patient's serum and the demonstration of the virus in the patient's sputum by inoculating it into mice are useful diagnostic procedures.

Lymphogranuloma Inguinale

Lymphogranuloma inguinale, also known as lymphogranuloma venereum and climatic bubo, is a venereal disease caused by a filterable virus. It is not in any way related to granuloma inguinale, the cause of which is uncertain (p. 428).

The virus of climatic bubo is 125–175 $m\mu$ in size. It can be cultivated in tissue culture and in the yolk-sac of fertile eggs. Laboratory animals are susceptible to it. The intradermal injection of the virus into the groin causes bubo in guinea-pigs, monkeys and dogs. Inoculated intracerebrally into guinea-pigs, monkeys, dogs, mice and cats, meningoencephalitis results. Elementary bodies are present in the affected tissue and pus (the granulocorpuscles of Miyagawa). The presence of cell inclusions have been reported in both animal and human lesions.

The disease is prevalent all over the world with greater incidence in the tropics. Transmission is by direct contact during sexual act. The virus displays a selective affinity for the lymphatic system. A primary sore develops but is often undetected. In the male a few days after infection bubos with multiple scattered foci of inflammation are developed in the groin. This is followed by suppuration and softening of the areas, forming circumscribed abscesses in the affected gland; they may eventually coalesce. Usually, they rupture through multiple points on the skin. As the primary lesion in woman is very often located in the vagina,

the infection spreads into the tissue around the anus and rectum, causing inflammation which later on leads to stricture of the rectum and elephantoid condition of the genitals esthiomene. Pelvic and other contiguous glands may also be enlarged.

The erum of convalescents possesses neutralising and complement-fixing properties.

The Frei test is very valuable in diagnosis. It is probably analogous to the tuberculin reaction. The antigen is prepared from a non-ruptured bubo. The pus is diluted 1 : 5 with normal salt solution and sterilised by heat at 60° C. for two hours followed by one more sterilisation for one hour on the succeeding day. We have found dilution with 0·5 per cent. phenol saline more convenient and equally efficient. When preserved at low temperature, the antigen keeps with unimpaired potency for a year or longer. The dose is 0·1 ml. injected intradermally. A control injection with the diluent employed is also given at a distance. In positive cases, a characteristic reaction, consisting of erythema, localised induration or papule of at least 1 cm. in diameter and often a vesicle at the puncture point, is developed in about 24–48 hours and may persist for a week or even longer. The Frei test is positive in a very high percentage of cases of lymphogranuloma inguinale; it may be negative in the very early stage. A positive reaction may be obtained long after healing. Positive reactions have been reported recently in cases of trachoma and other infections of the psittacosis group (p. 710). Frei's antigen may be prepared also from infected mouse brain and yolk-sac culture; lygranum is a commercial preparation from the latter.

Frei's antigen has also therapeutic value. Sulphanilamide compounds appear to be curative.

Rift Valley Fever

Rift Valley fever is a natural disease of sheep, occurring in epizootics. It was first reported from Rift Valley in Kenya, Africa. It causes heavy mortality among lambs, producing a severe type of necrosis of the liver. The virus is present in the blood, liver and spleen of the infected animal. Large number of nuclear inclusion bodies are present in the hepatic lesions. From sheep man may get the infection and develop a dengue-like disease. A high degree of immunity follows an attack in man or animals and their sera contain neutralising antibodies.

The disease is caused by a virus with an approximate size of 30 *mμ*. It stimulates the production of inclusion bodies. It is cultivable in chick embryo. The condition can be reproduced in sheep, cattle, monkeys and certain rodents. Transmission is probably by the agency of mosquitoes of the genus *Aedes*.

Mumps

Mumps, or epidemic parotitis, is an acute infectious disease most probably due to a virus which exhibits a selective affinity for the parotid glands.

The disease is universal in distribution and attacks more frequently children between the ages of five and fifteen years. It is highly contagious. Transmission is through droplet. It may also occur indirectly through fomites. In utero transmission has been reported. The patient is infectious for two to three days before the glandular swelling appears and the infectivity diminishes with the subsidence of the swelling. During this period, the saliva is infectious and by injecting filtrates of it through Stenson's duct an identical condition can be reproduced in monkeys. Further, with material from the infected gland the infection can be passed through monkeys in succession; after this serial passage it has been transmitted back to man.

The incubation period is 14–25 days. One or both parotid glands may be affected; even the other salivary glands may be involved. The virus invades the blood and complications like orchitis, and more rarely, ovaritis, mastitis and pancreatitis may follow. Fatality is exceedingly rare.

Immunity. Natural immunity to mumps does not seem to exist. Substantial and life-long immunity is conferred by an attack of mumps, whether one or both parotid glands are affected. Second attacks are very rare. The serum after recovery exhibits the capacity to neutralise the virus. It may be used for prophylaxis in the exposed subjects.

Dengue Fever

Dengue is a specific infectious fever, prevalent in the tropics and subtropics, probably due to a virus transmitted by the mosquito *Aedes aegypti*. It often occurs in epidemic form. Though symptoms are serious, fatality is very low.

The virus passes through Berkefeld filters. Successful transmission into guinea-pigs and cultivation in the membrane of the developing chick have both been reported. The virus is present in the blood for a day before and for three days after the onset of the disease. Blood taken at this time and inoculated either intravenously or subcutaneously into man produces the disease. Mosquitoes sucking blood during this period also become infected. After the infectious feed, the mosquito takes about eleven days to become infective and remains so for life. Attempts to reproduce the disease in experimental animals have been futile.

Nothing definite is known about the natural or acquired immunity. The latter may last for one year or more.

Sand-Fly Fever

Sand-fly fever, also called phlebotomus fever, papataci fever, and three-day fever, is a mild specific infectious fever of the tropical and sub-tropical regions caused by a virus and spread by *Phlebotomus papatasii* and possibly other species of sand-flies. It runs a short course of three days. The disease is not fatal and recovery is followed by a high degree of immunity.

The virus is about 160 $m\mu$ in diameter. It can be cultivated in the fertile chick embryo. It is present in the blood for twenty-four hours before and twenty-four to forty-eight hours after the onset of the disease, and sand-flies sucking blood during this period become infective in seven or eight days and remain so for life. The disease can be transmitted to human volunteers with infected blood. Experimental animals are not susceptible.

Warts or Verrucae

Warts are small benign epithelial tumours, formed by an overgrowth of the prickle-cell layer of the skin, with or without hyperkeratosis, and caused by a filterable virus. The condition is infectious. Both cytoplasmic and nuclear inclusions have been demonstrated. The condition can be reproduced in man by the intradermal inoculation of filtrates of materials from warts: it is possible to do so in succession. The incubation period is long. Infectious warts due to virus are found also in several species of animals.

Molluscum Contagiosum

Molluscum contagiosum consists of small tumour-like formations on the skin caused by a filterable virus. It is infectious and spread probably by contact or by the agency of fomites. The nodules are pea-sized, dome-shaped, shining, brownish or whitish in appearance, each with a central depression conspicuous in the later stages. They occur anywhere on the skin and more frequently in children. They may persist for months or years and then disappear spontaneously.

The molluscum nodules are produced by the local proliferation and hyperplasia of the epithelial cells. Degeneration and softening may set in later. Cytoplasmic inclusions, described by Lipschutz and called mulluscum bodies, found in the central cells are a striking feature in the histology of the condition. They are acidophile and appear as large oval bodies. Elementary bodies have been shown in materials stained by the Giemsa or Paschen stain. Successful transmission to man by injection with filtrates of materials from molluscum nodules has been claimed.

Trachoma

Trachoma is a specific infectious disease, characterised by follicular inflammation and papillary hypertrophy of the conjunctiva. If untreated, it leads to scarring and deformity of the eyelids and to considerable impairment of vision from "pannus" formation and corneal ulceration. The disease is more common in the poor and hygienically backward communities. Immunity to trachoma is transient.

The aetiology of trachoma is still uncertain. That trachoma is due to a filterable virus, is more than probable. A virus with an approximate size of 200 $m\mu$ has been isolated. Characteristic cell inclusions, called Halberstaedter-Prowazek bodies, are constantly present in the epithelial cells of the lesion. They are stained blue with Giemsa's stain and in the blue mass are also seen red granules. The latter are regarded as elementary bodies. Successful transmission to man has been claimed. A rickettsial aetiology has also been suggested, but the evidence for this is less satisfactory than that for the virus aetiology. *Bact. granulosis*,

a small, motile, Gram-negative bacillus isolated from trachoma cases by Noguchi (1925) and for a time widely accepted as the cause of the disease, is constantly present in the lesions. Recent reports show that trachoma, in its earlier stages, responds to sulphonamide theraby.

Inclusion Conjunctivitis

Inclusion Conjunctivitis is a specific infectious disease of the eye, characterised by an acute follicular inflammation of the conjunctiva. Two types of inclusion conjunctivitis have been described: inclusion blenorrhoea and swimming bath conjunctivitis. The latter is a misnomer as the same condition can be acquired independently of swimming bath. Both types are due to the same virus and in both cytoplasmic basophile inclusions are present in the epithelial cells of the affected conjunctiva. These inclusions resemble the cell inclusions of trachoma and with Giemsa's stain appear as bluish stained bodies containing red stained granules. The latter are probably elementary corpuscles. As in trachoma, there is also in inclusion conjunctivitis papillary hypertrophy of the conjunctiva, but neither corneal involvement nor scarring occurs in the latter as in the former.

The virus has not been cultivated. It resembles the virus of trachoma but is not identical with it. Experimental transmission to man and monkeys has been reported. The virus is found in the genito-urinary tract where a mild form of inclusion urethritis in the male and inclusion cervicitis in the female may be caused. Such carriers contaminate water and transmission is by bathing in such infected water. The disease may also be spread by contact or by means of fomites and independently of swimming baths. In the infants' type, transmission occurs during the passage of the foetus through the birth canal. In this, superinfection with the gonococcus may happen. Nothing definite is known about immunity in this disease.

Glandular Fever

Glandular fever, or infectious mononucleosis, is an acute infectious disease occurring in small epidemics. A characteristic feature of the disease is the enlargement of the anterior cervical glands. Lymphocytosis is present in most cases. The aetiology of glandular fever is not definitely known; probably it is a virus.

Experimental transmission of the disease by injecting filtrates of blood into monkeys has been claimed.

A curious phenomenon observed in glandular fever is the development of agglutinins in the blood against sheep's erythrocytes. They persist even after recovery. They are of the heterophile type and formed not against any specific antigen. Paul-Bunnell test, which is based on the occurrence of these agglutinins, is, therefore, a non-specific reaction; nevertheless it is diagnostic. It is done with the patient's serum heated at 55° C. for twenty minutes. Doubling dilutions of the serum are made in small test tubes in the usual way with normal saline, using 0·5 c.c. as the unit volume. Equal volumes of a one per cent. suspension of sheep's erythrocytes, prepared as in the Wassermann test, are added, the racks are shaken well and incubated at 37° C. for four hours. A saline control with no serum is also included. Normal serum may agglutinate sheep cells in dilutions up to 1 : 128; hence a positive reaction in this or a lower titre has not much value. In really positive cases, repetition will give positive results in increasing titres. Another point that should be kept in mind when interpreting the result is the occurrence of positive reactions in individuals treated with horse serum. Horse serum contains a heterophile antigen and this stimulates the production of its antibody in man which agglutinates sheep's erythrocytes. A difference between the agglutinins occurring in infectious mononucleosis and those in normal serum and in serum sickness is that both the latter can be absorbed out with guinea-pig's tissues, but not the former.

Infective Hepatitis

Infective hepatitis is an acute infectious disease, characterised by fever and usually jaundice. Available evidence indicates that the causative agent is a filterable virus. The disease is of recent discovery; usually it occurs in small epidemics.

The method of transmission is unknown; it is probably by ingestion. The incubation period is 20–40 days. The infective agent, which is very thermolabile, is present in the blood, duodenal washings and urine up to 48 hours after the onset of jaundice. Successful transmission of the disease has been reported in man. It also appears that a virus grows in the chorio-allantoic membrane of the developing chick after inoculation with filtrates of duodenal washings and urine. The liver is the chief seat of

lesion and jaundice is due to hepatitis and not to any duodenal catarrh or obstruction of the bile duct. A diffuse or zonal inflammatory reaction of the liver is the outstanding picture under the microscope.

A condition, clinically and histologically indistinguishable from the above, sometimes occurs within two to four months after the administration of measles convalescent serum, yellow fever vaccine containing human plasma and pooled adult serum. Its cause is obscure.

Primary Atypical Pneumonia

Primary atypical pneumonia, variously called virus pneumonia, acute pneumonitis or acute interstitial pneumonia, is an acute infectious disease of relatively mild nature. It may occur in epidemics as well as sporadically. Its aetiology is not definitely known; no bacterial agent has been recovered from the condition. An identical condition may be caused by the viruses of psittacosis and lymphocytic chorio-meningitis, but it seems to be distinct.

An interesting feature of the condition is the development of cold agglutinins against the Group O erythrocytes. The sera of patients suffering from other forms of pneumonia may also agglutinate the Group O cells but only in very low titre. The agglutinin titre of the patient's serum begins to rise in the second week of disease and persists for a month or more. The reaction may help in diagnosis. The test is done as in Paul-Bunnell test (p. 717), substituting the O group erythrocytes for sheep cells and exposing the racks to 0°–4° C. for one hour instead of at 37° C.; at the higher temperature the reaction is reversible. Agglutination at a titre of 1 : 80 may be taken as suggestive. A point to be remembered is that cold agglutinins are readily absorbed by red cells at low temperatures and so if the specimens have been exposed to cold, the temperature should be raised to above 20° C. before separating the serum for test. Otherwise, a positive serum may give apparently negative results.

CHAPTER XXXVII

BACTERIOPHAGE

Bacteriophage is a self-propagating lytic agent living on bacteria and capable of causing their dissolution. The exact nature of this lytic factor is not definitely known. It was discovered independently by Twort (1915) and d'Herelle (1917) and is often referred to as the *Twort-d'Herelle phenomenon* or the *phenomenon of transmissible lysis.* Bacteriophage is often referred to more briefly as *phage.*

While working with vaccine lymph, Twort observed that some colonies of staphylococci, developed on agar media, underwent on further incubation certain peculiar changes. The colonies became glassy and transparent in appearance and on microscopical examination of smears from them it was found that the staphylococcus had disappeared, being replaced by an amorphous debris containing minute granules. He also noted that this lytic phenomenon could be reproduced in succession by inoculating fresh colonies of staphylococci with materials from the glassy residue. He further showed that this lytic principle was filterable.

d'Herelle's observations were first with fluid cultures. During his investigations on bacillary dysentery, he noticed that broth cultures of motion yielded filtrates which evinced lytic properties on *Bact. shigae.* Fresh cultures of Shiga's bacillus, when inoculated with the filtrate, became clear after a period of incubation. He also demonstrated that the lytic phenomenon could be passed serially from culture to culture, thus proving its capacity for self-propagation. After extensive research, d'Herelle came to the conclusion that a filterable virus having its natural abode on bacteria was responsible for this lytic destruction and that it was normally found in the intestines of man. Hence he designated it *Bacteriophagum intestinale.* He did not, however, agree that it was the same agent demonstrated by Twort. But now opinion is unanimous that both are same. Subsequent investigations have fully confirmed and extended the observations of Twort and d'Herelle.

Habitat. Bacteriophage is widely distributed in nature and may be found in all the environments where the host bacteria exist. It is found in the intestines of man and animals, both in health and diseased conditions, in sewage, water and soil. The isolation of it from blood and tissues has been reported. More than one species may exist in the intestine of the same individual. Members of the *Bacterium* genus, the cholera vibrios, the plague bacilli and staphylococci seem particularly prone to phage parasitisation. More than one race or strain of phage may live on the same bacterial species.

Nature. Recent studies have established that bacteriophages are particulate filterable agents capable of independent propagation. The majority of observers now agree that phage is a type of virus parasitic on bacteria. There can be no doubt that the phage displays close resemblance to the virus. It is generally agreed that an enzyme is responsible for the actual lytic process. But the source of this enzyme is in dispute, whether it is produced by a definite virus or by the bacteria themselves. The real issue is whether phage is a living virus or a non-living enzyme. Its living nature has not been irrefutably proved, but the evidence in support of it far outweighs that against it. The lytic factor undoubtedly increases in amount. Those who uphold that it is a non-living agent postulate that it is an autolytic enzyme, set free by the disintegrating bacteria and that it can even in minute traces set up dissolution of growing bacteria liberating large quantities of the enzyme, thus giving rise to a semblance of multiplication and increase.

Types. d'Herelle considered that there was only one type of phage capable of adapting itself to different species of bacteria. But nearly all other observers are now agreed that there are several species of phages, probably as many as there are bacterial species. The results of immunoligical studies are also in harmony with this view. Again, a species of phage may show a multiplicity of serological races, for example the cholera phage.

Phage exhibits a high degree of species specificity. For instance, the cholera phage will act only on *V. cholerae* and not on the colon bacillus. This would seem to depend upon the antigenic structure of the bacterial species. A phage that is lytic to one species may, however, act on related species or even on unrelated species, though the action may not be marked on the

latter. This is considered to be due to the presence of common somatic antigens in the structure of the different susceptible bacterial hosts. For example, certain *Salmonella* species with common somatic antigens are lysed by a single phage. It is also possible to acclimatise a phage to a different bacterial species by serial passage.

General Properties. Bacteriophage, like the virus, is ultramicroscopic and filterable. By ultrafiltration their size has been found to vary from 8–75$m\mu$. Pure cultures are markedly uniform in size. As shown by the electron microscope, some phages have a cubical shape, while others are round. Again, some have a tail-like process, recalling the appearance of spermatozoa. Phage particles carry a negative charge as do the viruses and bacteria. Their metabolic activities have not been much studied as it is not possible to grow them apart from bacteria. Limited investigations, however, reveal no detectable metabolic activity. They display varying degrees of resistance to physical and chemical agents. In general, their resistance to these agents is slightly greater than that of the non-sporing bacteria. Heat inactivates phage after an oxposure at 75° C. for thirty minutes or more. Ultraviolet rays are rapidly lethal to phage. Desiccation is resisted for long. The phage remains viable at freezing temperature and withstands the action of glycerine, alcohol and one per cent. phenol. Sealed in tubes and preserved in the dark, phage remains active for years.

Phage Activity. A complete clearing of growth is the characteristic feature of phage activity. Marked difference in the activity is encountered among the various phages; some are very powerful, retaining their potency even in very high dilutions, $1:10^9$–$1:10^{10}$. Phage grows and multiplies best on young actively growing bacteria. Neither growth nor lysis occurs on dead organisms which can however adsorb phage. The mechanism of lysis, according to d'Herelle, consists in the penetration of a phage into the bacterial cell, rapid multiplication, distension of the cell and its explosive bursting and disappearance, leaving a granular debris. Numerous phage particles are seen within the swollen cell; on bursting these are liberated into the surrounding medium. The lytic activity on organisms growing on solid media is marked by a clearing of the growth, leaving circular areas showing colourless, glassy, residual structures called *plaques*.

These plaques are regarded as colonies of phage. Later bacterial colonies may develop in the clear areas. If the lytic principle is added to a young broth culture of a susceptible organism, complete clearing results after a period of incubation and few, if any, bacteria can be found on microscopic examination. If it is reincubated, growth and turbidity reappear. This is due to the fact that a few sturdy organisms in the primary culture, having withstood the action of phage, multiplied during the second incubation. Such cultures are referred to as *secondary cultures* and the resistant strains as *lysogenic*, because though resistant to its action they are still parasitised by phage. They are really phage carriers. This can be proved by filtering such cultures when the filtrates are found lytic to young susceptible cultures. Such resistant strains exhibit another peculiarity in that they have become rough variants, losing their original antigenic structure and virulence. In fact, phage action is now well recognised as a potent means of inducing bacterial variation (p. 344).

Isolation. Phage may be isolated from faeces, sewage, water or pus. Ordinary nutrient broth, adjusted to pH 7·6 and contained in large flasks, is inoculated with relatively large amounts of faeces, made into a uniform emulsion. The flasks are thoroughly shaken and incubated aerobically at 37° C. for 18–24 hours. The cultures are then filtered through Seitz filters. The filtrates contain the phage.

The presence of phage in the filtrate can be demonstrated by its lytic action on bacterial growth. For this purpose, a young broth culture of an organism, for instance *Bact. shigae*, is sown uniformly on an agar plate; after the inoculum has dried, a loopful of the filtrate is transferred to one or more spots over the seeded media which is then incubated. After an interval of incubation, it will be found that a sheet of growth has developed, showing clear circular zones at points where the medium has been superinoculated with the filtrate. The organisms in these clear patches have been lysed by the phage.

The filtrate may contain many types of phage. In order to isolate a pure culture, several high dilutions are made with the filtrate. If a cholera phage is the one desired, a dense emulsion of *V. cholerae* is made from an agar growth and sufficient of it is added to each of the dilutions so as to cause a distinct turbidity in them. Loopfuls from each of these dilutions are then

uniformly spread on separate agar plates and incubated. After 18–24 hours, small discrete plaques will become visible on some of the plates. Subcultures are made from these individual plaques into broth tubes previously inoculated with *V. cholerae*. After a further incubation, the subcultures are filtered and it will be found that the filtrate contains a fairly pure cholera phage. If further purity is aimed at, the above process is repeated two or three times when a final product of high purity is obtained. Concentration of phage can be effected by the enrichment method; a few drops of an actively growing culture are added to a large amount of dilute phage, incubated for 18–24 hours and filtered. This may be repeated if necessary.

Antigenic Structure. Bacteriophage has a definite antigenic structure, displaying a high degree of specificity. Rabbits inoculated with bacteria-free phage yield an antiphage serum which neutralises the phage, inhibiting its lytic property. Such sera may also agglutinate phage particles. A serum produced against one species of phage interacts only with that species and not with others. The mechanism of the action of antiphage sera is little understood.

Clinical Value. d'Herelle regarded phage as playing an important natural role in the process of recovery from infectious diseases. But there is no evidence whatsoever to support this view. It is true that in many infectious diseases the presence of phage can be recognised. But it has not been shown that such naturally occurring phage does materially alter the course of a disease. Phage may induce dissociation in bacteria in the body. Whether this has any significant influence on the termination of infectious diseases, is an interesting point for search.

Bacteriophage is harmless to the tissues. Considerable divergence of opinion exists about its therapeutic value. It has been used both prophylactically and therapeutically, but the results have not justified the high expectation entertained of its clinical value at the time of its discovery. The remarkable lytic activity occurring in the test tube does not occur in vivo; why it does not occur, is still a mystery. In the tissues, the environment is probably not favourable for the lytic action to take place or the environment may be in some way inactivating the phage.

For treatment purposes, the local application of phage, wherever possible, is to be preferred as in the case of staphylo-

coccal and streptococcal lesions. The phage may be incorporated into wet dressings or instilled into cavities such as infected nasal sinuses, middle ear and bladder. Only a phage of proved potency should be used. It must be applied at least once or twice a day. The use of all antiseptics should be stopped since they inhibit the activity of phage. In intestinal infections, like cholera, enteric and dysentery, it is administered orally (p. 476). Phage has also been given subcutaneously, intravenously and intraspinally; but the results obtained are equivocal.

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